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THE UNIVERSITY OF ALBERTA

REGULATION BY ANDROGENS OF THE GROWTH AND FUNCTION OF THE RAT PROSTATE

by

© BARRY H. LESSER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "REGULATION BY ANDROGENS OF THE GROWTH AND FUNCTION OF THE RAT PROSTATE" submitted by BARRY LESSER in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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DEDICATION

I would like to dedicate this Thesis to my parents, Mr. and Mrs. Samuel Lesser, for their encouragement and interest in the pursuit of this work.

ABSTRACT

The response of the rat prostate to deprivation and restoration of androgens has been studied with the objective of gaining further knowledge about regulation of tissue atrophy, cell proliferation, and differentiation.

Castration of an adult male rat results in cessation of DNA synthesis and cellular renewal in the prostate within 24 hours. Rapid loss of cells from the tissue commences about 3 days after castration and proceeds until the sixth day, when about 15% of the cellular complement remains; after this time further loss of cells occurs very slowly. No evidence has been found to indicate that loss of cells is a selective process.

The response of the prostate to hormone administration has been characterized using rate of incorporation of thymidine and changes in number of nuclei and wet weight as indices of rate of cellular proliferation and of functional capacity of the tissue. This system provides a biochemical assay for testing the potencies of various androgens and antiandrogens and the possible role of mediators of androgen action.

The response of the castrate rat prostate to restoration of androgens depends on the number of cells present in the tissue. If this number is normal, as in short

term castrates, androgens stimulate secretion (the differentiated function of the prostate) but not DNA synthesis or cell division. Long term castrates, whose prostates contain less than the normal cellular complement, respond to restoration of hormone by proliferating until the number of cells has returned to normal. Proliferation is preceded by a period of 1-2 days during which no DNA synthesis or cell division occur; the duration of this lag period does not seem to be affected by the length of time between castration and initiation of treatment. All the cells that survive castration appear to participate in the regeneration of the prostate. Once proliferation has restored the normal cellular complement, DNA synthesis and production of cells are turned off. This limitation on prostatic size does not appear to be due to depletion by the cells of some substance essential for division, nor to loss of response to hormone, since secretion continues to be stimulated. It is concluded that there exists in the prostate an internal homeostatic mechanism that limits the number of cells in the prostate to the normal number. If the cellular complement is smaller than normal, androgens are necessary for its restoration, but they cannot override the internal control mechanism.

ACKNOWLEDGEMENTS

I would like to offer my sincere thanks to my supervisor, Dr. Nicholas Bruchovsky, for his invaluable direction, advice and criticism during the course of experimental work and preparation of this thesis. Through sharing his knowledge and experiences I feel that I have gained immeasurably both as a scientist and as a person.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
BSA	bovine serum albumin
Ci	curie
cpm	counts per minute
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
exp	exponent to the base e
g	gram
xg	centrifugal force relative to gravity
l	litre
ln	logarithm to the base e
M	molar
min	minute
N	normal
nm	nanometer
P	probability
PBS	phosphate buffered saline
pg	picogram
RNA	ribonucleic acid
S.E.	standard error (of the mean)
uCi	microcurie
um	micron
v/v	volume per volume
w/v	weight per volume

I. INTRODUCTION [1]

Primitive, single celled organisms are adapted to grow at the fastest rate possible subject only to the limitations of the existing environmental conditions. For these organisms the process of cellular division is also the means by which the species is propagated and thus in a teleological sense a rapid growth rate is designed to ensure survival of the species. Because these organisms are single celled, each cell must have the capability of performing all the functions required for survival of the organism. As one moves up the evolutionary scale, perhaps the most striking change observed is an increasing tendency towards specialization, or differentiation, of cells so that any given cell performs only a small part of the functions necessary for the viability of the whole organism and in turn depends on the functions performed by other cells for its own survival. This type of organization of multicellular organisms introduces the need for cooperation, or communication, among individual cells. One aspect of functional specialization of cells is that the process of cell division has lost its reproductive function; instead it

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[1] Because of the wide range of material covered in this introductory chapter, few specific literature references are quoted. Footnotes at the beginning of each section designate reviews from which these references can be obtained. References dealing specifically with the experimental system are cited in the introduction of each chapter.

serves to establish the appropriate numbers of cells in various tissues during development of the adult animal, and then to maintain these numbers within the limits conducive to most efficient functioning of the tissue within the body as a whole. The problem as to how cellular proliferation and differentiation are regulated is one of the main ones of biology today. For reasons which will be outlined below, the response of the rat prostate to androgenic hormones has been chosen as a model system for the study of this problem.

PROLIFERATION AND DIFFERENTIATION [1]

The development of a multicellular adult animal from a single oocyte is a process that can be reduced in its basics to a series of cellular divisions accompanied by increasing differentiation of the progeny cells to functional tissues.

The key to regulation of this complex process is undoubtedly intercellular communication. In the early stages of development, when semi-differentiated precursors of organ systems are emerging, spatial relationships among cells appear to be the main factor, and communication between cells is probably direct. At later stages indirect or long distance communication mediated by factors such as hormones becomes very important, and continues to do so into adult life.

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[1] For reviews, see Baserga (1971), Cameron and Thrasher (1971), Cameron et al (1971).

It is now well established that functional specialization of cells does not result from loss of genetic information, but rather from restricted expression of this information, since under certain conditions differentiated single cells of certain organisms are capable of regenerating an entire organism. Thus in order to understand differentiation it is ultimately necessary to determine how gene expression is regulated. Considerable progress has been made in this area, but it is not yet possible to reduce the problem to this level.

In the adult animal, tissues can be classified into three main types with regard to their proliferative status. Firstly, there are the tissues that are either incapable of proliferating, such as nervous tissue, or that proliferate very slowly, such as lung, cardiac muscle, and skeletal muscle. These tissues appear to be permanently differentiated. Secondly, there are the rapidly turning over tissues, such as skin, intestine, bone marrow, and seminiferous tubules of the testis. This latter group is generally characterized by the existence of a self-renewing "stem" cell population. This subpopulation proliferates continuously and provides a constant supply of differentiated cells to replace those lost as a result of functional turnover. The rate of proliferation in these tissues is matched to the rate of cell loss so as to maintain a constant tissue size. Hence in the case of skin,

for example, cell damage by wounding leads to an increased proliferative rate. Thirdly, certain tissues normally proliferate slowly but are capable of rapid proliferation in response to an appropriate stimulus, e.g., liver, uterus, mammary gland, prostate, and seminal vesicles. In the case of liver an appropriate stimulus is removal of part of the organ, while for the latter four organs the stimulus is hormonal. In these tissues the majority of the surviving cells is characteristically involved in the proliferative response, and proliferation generally stops once the normal functional size has been restored.

Proliferation is a cyclical process, with one cell giving rise to two genetically identical daughter cells. These daughter cells can re-enter the proliferative cycle and divide again, or they can differentiate. On a cytological basis, the cell cycle can be divided into two phases - mitosis, during which the chromosomes condense to recognizable structures and are partitioned between the two daughter cells and actual cell division occurs, and interphase. Within interphase there is a discrete period during which the DNA of the cell is replicated, called the DNA synthetic, or S, phase. This phase can be detected biochemically by incorporation of a labeled DNA precursor. The time between completion of mitosis and initiation of the next round of DNA synthesis is called G1, and the period between completion of DNA synthesis and onset of mitosis is

called G2. The durations of S, G2, and mitosis are relatively constant in different proliferating populations, being approximately 6-12 hours, 2-6 hours, and 1 hour respectively. The major variation in the total cell cycle time is in the duration of G1, which can vary from hours in the case of rapidly proliferating populations to days, months, or even years in slowly proliferating populations. Cells that have left the proliferative cycle and differentiated are considered to be in a phase called G0. In practice, however, it is difficult to distinguish cells in G0 from those with a very long G1 phase.

The relationship between proliferation and differentiation is one that has caused considerable discussion. As a general rule there seems to be an inverse relationship between the two, i.e., proliferating cells tend to be less differentiated and differentiated cells tend not to proliferate. However the relationship appears to be more one of degree than an absolute one. Another interesting concept that has emerged is that proliferation may in fact be necessary for differentiation since, in several systems studied, proliferation appears to precede differentiation, and inhibiting proliferation can inhibit differentiation. This concept is reasonable because the process of replication of the genome would provide an opportunity for rearrangement of the elements regulating gene expression.

REGULATION OF PROLIFERATION [1]

In terms of cell populations as a whole, two levels of regulation can be recognized: external regulation and internal regulation. External regulation is regulation by elements from outside the tissue and is generally stimulatory. Examples of external regulation include the action of various mitogenic hormones on their target tissues, such as androgens on seminiferous tubules or male sex accessory glands, estrogens on breast or uterus, isoproterenol on salivary gland, and adrenocorticotropic hormone on the adrenal cortex. Internal regulation is regulation by elements produced within the tissue being regulated. A logical way for a tissue to maintain a constant cell number would be a negative feedback control system in which each cell would produce inhibitors of proliferation that would act on the population as a whole. Loss of cells would result in decreased production of the inhibitor, thus freeing the remaining cells to proliferate. As the cellular number increased, the concentration of inhibitor would rise until it was high enough to inhibit further proliferation. Presumably this would occur when the number of cells had returned to the normal level. Such mitotic inhibitors have been described and have been called chalones (Bullough et al, 1967). They have been implicated in growth regulation of

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[1] For reviews, see Baserga (1971), Cameron and Thrasher (1971), Cameron et al (1971).

the epidermis, the granulopoietic and erythropoietic systems, and the liver. For many tissues it is quite possible that both external and internal levels of regulation are superimposed on one another.

In terms of the cell cycle, agents that stimulate proliferation can do so either by inducing previously nonproliferating cells to enter the cell cycle or by speeding up the progress of those cells already in the cycle through the cycle. In the first case, the growth fraction (that is, the fraction of cells in the population that is in the proliferative cell cycle) is increased, and in the second the cell cycle time is reduced. Most regulators of proliferation appear to have both effects. Because nonproliferating cells are generally arrested in G1 (that is, when stimulated to proliferate they must replicate their DNA before dividing), this stage of the cell cycle is considered to be the one during which the major regulatory events occur. Once cells have started DNA replication, they usually proceed immediately through S, G2 and mitosis provided RNA and protein synthesis are not inhibited. There has been a report (Gelfant, 1962) of mouse epidermal ear cells arrested for long periods of time in G2. When stimulated to proliferate these cells divide within a few hours without first replicating their DNA. However this phenomenon does not appear to be widespread.

Cancer is essentially a disease of unregulated growth.

It is now recognized that malignant cells do not necessarily proliferate faster than all normal cells, although malignant cells derived from a given tissue tend to proliferate more rapidly than normal cells from that tissue. The principal characteristic of malignant tissues is that the rate of cell production exceeds the rate of cell loss, so that in contrast to the normal state there is a constant increase in tissue mass. The neoplastic cells tend to be less differentiated than their normal counterparts. Usually it is still possible to recognize the tissue of origin, but the cells have lost to varying degrees the ability to perform the differentiated functions of that tissue. A second important characteristic of malignant cells is their ability to metastasize, or spread to tissues beyond their tissue of origin. It is quite possible that one of the defects in neoplastic cells is impairment of the ability to communicate among themselves and with normal cells.

Despite almost a century of research, however, the fundamental nature of the abnormality in malignant cells is still unclear. A major step towards elucidating this abnormality would be discovery of the mechanisms governing the proliferation of normal tissues, in order to provide a standard for comparison of mechanisms operating in cancer cells. For these types of studies it is definitely advantageous to work with a tissue whose proliferative status can be manipulated at will.

Among the external regulators of proliferation mentioned previously, the androgenic hormones are the chief regulators of the proliferation and differentiation of all the male sexual tissues except the testes and hence are the essential hormones for the development and maintenance of the primary male sex characteristics. At puberty the increased levels of androgens result in emergence of the secondary male sex characteristics, and sustained high levels of androgens are necessary for retention of these characteristics in the adult.

ANDROGENS AND PRIMARY MALE SEX CHARACTERISTICS [1]

During the earliest stages of embryological development, all embryonic structures necessary for the development of either sex are present. The indifferent gonad is composed of a primordial cortex and medulla. Both Wolffian ducts, the progenitors of the male urogenital tract, and Mullerian ducts, the progenitors of the female urogenital tract, are present.

The first step in sex differentiation is gonadal differentiation. In males, the medulla of the indifferent gonad expands, enveloping the germ cells, and the cortex regresses. In females, the gonadal cortex becomes

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[1] These aspects of androgen action have been reviewed by Burns (1961), Gallien (1965), Price and Ortiz (1965), Wells (1965) and Jost (1970).

predominant and incorporates the germ cells, and the medulla shrinks. The testis then differentiates rapidly while the ovary remains undifferentiated for some time. This stage of development is undoubtedly under genetic control with an uncertain factor originating from the Y chromosome probably responsible for development of the male gonad. In mammals, administration of sex hormones does not affect gonadal differentiation.

The course of subsequent development depends on the presence or absence of testes during several critical developmental stages. The testis secretes androgens, which are the same as the adult hormones. The androgens are responsible for development of the Wolffian ducts and the structures derived therefrom - epididymis, vasa deferentia, seminal vesicles, and prostate. There are certain critical stages in development during which androgens must be present, but after the critical stages, hormone is no longer necessary. In addition to androgens the testis also secretes some unknown substance that causes the Mullerian ducts to atrophy. In the absence of a testis, as in the case of females or neonatal castrates of either sex, sexual development follows the female course. The Wolffian ducts are lost and the Mullerian ducts give rise to the internal female genitalia - oviduct, uterus and vagina. These structures do not require hormone for their development but development is accelerated by estrogens. Androgens can

replace the testis insofar as development of the Wolffian ducts is concerned, but cannot cause atrophy of the Mullerian ducts.

The external genitalia also follow the female course of development in the absence of hormonal stimulus, but are more susceptible to hormonal effects than are the internal genitalia. Administration of female sex hormones to males inhibits penile development and stimulates mammary gland enlargement. Conversely, male sex hormones stimulate clitoral enlargement and inhibit breast development in females.

Neural structures mediating sexual behavior are patterned by the presence or absence of normal testes during a critical period just before or after birth, depending on the species. Presence of testicular hormone imposes the male pattern upon neural structures in the hypothalamus. In the absence of androgens the female pattern develops. Because the hypothalamus regulates pituitary function, these hormonal effects also result in the male pattern of gonadostimulatory activity of the adult pituitary or the cyclic female pattern.

The pituitary does not appear to be involved in the primary differentiation of sex. Early hypophysectomy has no effect on gonadal development. Hypophysectomy prior to development of accessory sex structures does not produce any

essential change in the course of differentiation, but development is retarded as a result of decreased gonadal secretory activity due to lack of gonadotropic stimulation.

ANDROGENS AND SECONDARY MALE SEX CHARACTERISTICS [1]

The male reproductive system remains in an immature state until the time of puberty, when pituitary gonadotropin secretion begins. Follicle stimulating hormone (FSH) acts on the seminiferous tubules to induce and maintain spermatogenesis. Interstitial cell stimulating hormone (ICSH) causes testicular Leydig cells to differentiate and to secrete testosterone. It is this stimulation of testosterone secretion that results in development of secondary sex characteristics such as growth of facial and body hair, enlargement of external genitalia, lowering of voice, recession of scalp hair, growth spurt, libido and sexual potentia. In addition, testosterone is involved along with FSH in stimulation of spermatogenesis. Finally, testosterone produces enlargement and development of secretory activity of the accessory sex organs, such as prostate and seminal vesicles. All these effects can be produced prematurely in immature animals by administration of androgens.

All the secondary sex characteristics are reversible,

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[1] For review see Paulsen (1962).

and in the adult require the continuous presence of testosterone for their maintenance. Thus, for example, castration or administration of antiandrogens results in atrophy of the prostate and seminal vesicles and hormonal restoration stimulates regeneration of these organs. Hence these tissues provide a readily manipulable system for the study of proliferation and differentiation.

STRUCTURE AND FUNCTION OF THE PROSTATE [1]

The prostate is the only male sex accessory gland common to all orders of mammals. However the anatomical and histological structure and chemical composition of this organ vary markedly between orders, and sometimes even between families within orders. In addition, the prostate of a given species often contains regions which differ greatly from one another. For example the rat prostate comprises three paired lobes - ventral, dorsal and lateral - which differ in histological structure, chemical composition and response to hormonal stimuli. The human and canine prostates are not lobed, but within the glandular body can be distinguished two distinct histological zones, the central zone and the peripheral zone. Thus direct extrapolation of information about the prostate between species is not valid. Nevertheless, the basic functions and control mechanisms

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[1] Reviewed by Price and Williams-Ashman (1961) and by Schoonees (1971).

among various species are probably quite similar, with differences generally quantitative rather than qualitative. The organ studied in this thesis is the rat ventral prostate, but the discussion that follows will concentrate on the aspects of prostatic structure and function common to all species, unless otherwise mentioned.

All prostates consist of a number of alveoli composed of a secretory epithelium surrounding a lumen (Moore et al, 1930). Ducts leading from the alveoli empty into the urethra. The epithelium is typically columnar. Nuclei are located at the base of the cells and have conspicuous nucleoli and chromatin particles. There is a supranuclear clear zone or light area in the cytoplasm corresponding to the Golgi zone. The alveoli are surrounded by a continuous basement membrane resting on a stroma of connective tissue containing smooth muscle and blood vessels. Electron microscopy reveals a structure of a typical secretory cell, with considerable rough endoplasmic reticulum, a conspicuous Golgi complex, and the apical membrane forming numerous microvilli extending into the lumen.

The only known function of the prostate is to secrete a part of the seminal plasma. Substances found in appreciable quantities in the prostatic secretion include zinc, fructose (rat dorsal prostate and coagulating glands only), the polyamines spermine and spermidine, citrate (rat and human), amino acids, prostaglandins, various small proteins, and

several enzymes, most notably acid phosphatase and alkaline phosphatase.

ENDOCRINE CONTROL OF THE PROSTATE [1]

All aspects of prostatic function are under the direct control of androgens. Castration of an adult animal results in a general decline in metabolic activity of the prostate followed by decreases in cell size and loss of cells (see Chapter III). These effects can be prevented or reversed by administration of androgens (see Chapter IV).

In addition to androgens, several other hormones have effects on the prostate, either directly or indirectly by affecting the levels of androgen available to the prostate.

Estrogens can either inhibit or stimulate the prostate, depending on several factors including age and species of the animal, the part of the prostate studied, the parameter of prostatic function measured, the type, dose and duration of estrogen administration, and the androgenic status of the animal. In normal males the predominant effect of estrogen administration is atrophy of the prostate. This is an indirect effect due to feedback inhibition of pituitary gonadotropin secretion, resulting in failure of the testes to produce androgens, i.e., essentially pharmacologic castration. In castrate animals or in organ culture,

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[1] For review, see Schoonees (1971).

estrogens stimulate fibromuscular tissue and produce hyperplasia and squamous metaplasia of epithelial cells. However secretory activity of the epithelium is never stimulated by estrogens. In cases where estrogens and androgens are present simultaneously, the overall effect depends on the relative levels of the two.

Several pituitary hormones have effects on the prostate. The gonadotropin ICSH has major indirect effects by stimulating the Leydig cells of the testis to secrete testosterone. Both prolactin and growth hormone appear to act directly on the prostate. Although neither has very marked effects when administered alone to hypophysectomized-castrated animals, both hormones potentiate the action of testosterone in stimulating prostatic growth and function. This synergistic effect could possibly be mediated at the level of uptake or metabolism of androgens by the prostate.

Both from studies in alloxan-diabetic rats and in organ culture, insulin also appears to have direct effects in augmenting testosterone action on the prostate.

The adrenal glands secrete small quantities of androgens that are insignificant relative to normal testicular production. However, output of adrenocorticotropic hormone (ACTH) rises following castration due to loss of feedback inhibition by androgens, and the resulting increased production of adrenal androgens may have

detectable effects on the prostate in this situation. The major hormones secreted by the adrenals, the corticosteroids, may possibly have minor effects in modulating androgen action.

Thyroid status of the animal affects prostatic growth and function, probably as a result of the effects of thyroid hormones on metabolism and excretion of testosterone. However no direct effects on the prostate have been demonstrated.

Hence, despite the multiplicity of hormones that have effects on the prostate, the inescapable conclusion is that the growth and function of the prostate essentially depend exclusively on the androgenic status of the animal. This fact, plus the apparent homogeneity of cell structure and presumably hormonal response, and the ready availability of sufficient quantities of tissue for biochemical studies make the androgen-stimulated rat prostate an excellent system for the study of the regulation of cellular proliferation and differentiation. In addition, the existence of two common abnormal growth conditions of the prostate greatly enhances the usefulness of any information derived from this system.

ABNORMAL GROWTH CONDITIONS OF THE PROSTATE [1]

Statistics indicate that prostatic carcinoma is the third most common cause of death from neoplastic disease in men in the United States (American Cancer Society, 1970), and the condition of benign prostatic hyperplasia occurs almost universally in aging men. Unfortunately the occurrence of these conditions is confined to man, and no completely homologous animal models exist, although canine prostatic hyperplasia has characteristics similar to human benign prostatic hyperplasia.

In man, prostatic carcinoma and benign prostatic hyperplasia arise in different regions of the prostate - benign prostatic hyperplasia in the periurethral, or central, region of the gland and carcinoma in the peripheral segments of the prostate. In benign prostatic hyperplasia the number of cells is increased but they do not seem to be abnormal. The condition does not appear to be precancerous and clinical problems arise only if the enlarged prostate obstructs urinary flow. Prostatic carcinoma cells, on the other hand, are characteristic of neoplastic cells in that they multiply in an uncontrolled manner and metastasize to other tissues, although in early stages they may often resemble adult prostatic epithelium rather than undifferentiated tissue.

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[1] For review, see Price and Williams-Ashman (1961) and Schoonees (1971).

Androgens appear to be involved in both benign prostatic hyperplasia and prostatic carcinoma, but almost certainly are not the sole causative factors. Benign prostatic hyperplasia is not observed in men castrated early in life, but castration or estrogen administration have little effect once the condition has developed, and androgen administration in later life does not increase the incidence of the condition. The majority of prostatic carcinomas, on the other hand, initially respond dramatically to castration or estrogen treatment but eventually recur and begin to grow again. This relapse may be due to change of cancerous cells to hormone independence and/or to selection of hormone independent cells. Androgens alone apparently cannot induce prostatic cancer, since they do not increase its incidence in man or induce it in dogs. Their effects on established tumors are variable. Prostatic tumors can, however, be induced in rats and mice by administration of chemical carcinogens such as benzpyrene or methylcholanthrene. Some of these are androgen dependent and their incidence is increased by simultaneous administration of estrogen but reduced by administration of androgen.

The above findings indicate that androgens play an important role in both benign prostatic hyperplasia and prostatic carcinoma, but that additional factors are certainly involved. The understanding of the role of androgens in abnormal growth conditions would be greatly

clarified by elucidation of their role in regulating the growth and function of normal target tissues. With this end in mind, this thesis deals with the characterization of the various responses of the adult rat ventral prostate to hormonal manipulation and with the mechanisms regulating prostatic growth and function.

II. MATERIALS AND METHODS

INTRODUCTION

General methods used throughout this study are outlined in this chapter. Special procedures used in particular aspects of this work will be described in the "Materials and Methods" section of the appropriate chapter.

MATERIALS

General laboratory chemicals and reagents were purchased from Fisher Scientific Company (Montreal, P.Q.) or from Sigma Chemical Company (St. Louis, Mo.), unless specified otherwise. Ultra-pure sucrose was purchased from Schwarz/Mann (Orangeburg, N.Y.) and steroids were obtained from Steraloids, Inc. (Pawling, N.Y.). All water used was glass distilled and organic solvents were double glass distilled.

Phosphate buffered saline (PBS), containing 0.14 M sodium chloride, 2.7 mM potassium chloride, 0.9 mM calcium chloride, 0.5 mM magnesium chloride, 8.1 mM monobasic sodium phosphate, and 1.5 mM dibasic potassiur phosphate, was prepared after the method of Dulbecco and Vogt (1954). Tris buffer, pH 7.0, contained 0.01 M Tris (hydroxymethyl

aminomethane), 0.05 mM ethylenediamine tetraacetic acid (EDTA), 5 mM magnesium chloride, and 0.5 mM mercaptoethanol in water, adjusted to pH 7.0 with hydrochloric acid at room temperature using a Radiometer pH meter (Copenhagen, Denmark).

RADIOCHEMICALS

[Methyl-^{3}H] thymidine (45-55 Ci/mmole) or [methyl-^{14}C] thymidine (55mCi/mmole) were purchased from New England Nuclear (Boston, Mass.). Purity was checked monthly by thin layer chromatography on phosphoethyleneimine-cellulose plates (Brinkmann Instruments, Rexdale, Ont.) using water as the mobile phase and was considered acceptable only if thymidine plus thymine accounted for greater than 90% of the radioactivity.

Liquid scintillation counting of aqueous samples was carried out using a Bio-solv cocktail containing 6 g of 2,5-diphenyloxazole (Amersham-Searle Corp., Arlington Heights, Ill.), 216 g Bio-solv (BBS-3; Beckman Instruments, Fullerton, Calif.) and 75 ml water per litre of scintillation grade toluene (Fisher). Samples were deposited in glass vials (Value Vials; Beckman) and radioactivity measured in a Beckman LS-250 automatic liquid scintillation system. External standardization was used to convert cpm to dpm, and efficiency of ^{3}H counting was about 30%.

DNA DETERMINATION

DNA was measured using the diphenylamine procedure of Burton (1956) with calf thymus DNA (Sigma) as standard. Color development was allowed to proceed at 30° for 16-20 hours and optical density at 600 nm was determined with a Spectronic 20 colorimeter (Bausch and Lomb, Rochester, N.Y.).

EXPERIMENTAL ANIMALS

Adult male Wistar rats (270-340 g) were purchased from Woodlyn Laboratories (Guelph, Ont.) and maintained in the Health Sciences Animal Center on a diet of Rockland rat chow and water ad libitum.

Orchiectomy was performed via the scrotal route under ether anesthesia.

Stock solutions of hormones were stored in 95% ethanol. Just prior to injection the hormone solution was diluted ten fold with 10% (v/v) polyoxyethylene sorbitan monopalmitate (Tween 40; Sigma). At the doses of hormone employed this dilution generally resulted in formation of a fine precipitate. Animals under light ether anesthesia were injected subcutaneously with 0.2 ml of this suspension per 100 g body weight using a 25 gauge needle. Sites of injections were varied during the course of treatment. All injections were done between 9:30 and 10:30 a.m.

Injections of other materials in sterile saline solutions were made intraperitoneally using a 22 gauge needle.

At the appropriate time animals were killed by decapitation. Prostates were removed, stripped of connective tissue, placed in a beaker on ice, and weighed. Three to seven prostates were pooled for each experiment.

ASSAY FOR RATE OF DNA SYNTHESIS

In vitro. The prostates (250-900 mg) were chopped with a Sorvall TC-2 tissue sectioner (Sorvall, Norwalk, Conn.) and washed with ice cold PBS. The tissue was resuspended in 5 ml Minimal Essential Medium (Grand Island Biological Co., Berkeley, Calif; Catalogue number F-11) at 37° C and incubated with gentle shaking in an atmosphere of 5% carbon dioxide, 95% oxygen (v/v). After 5 minutes of temperature equilibration 5 ml of the above medium containing 10 uCi [methyl-^{3}H] thymidine/ml were added to give a radioactivity concentration of 5 uCi/ml and a thymidine concentration of 0.02 mM in a total volume of 10 ml. Incubation was continued for a further 20 minutes. Incorporation was terminated by pouring the suspension over crushed ice and diluting it with ice cold PBS. The suspension was centrifuged in a Sorvall GLC-1 centrifuge (HL-4 rotor, Rav 12.5 cm) at 400xg for 5 min and the tissue washed once with ice cold PBS. Nuclei

were isolated as described below and sonicated using a Bronwill Biosonik III (Bronwill Scientific, Rochester, N.Y.) with two 10 second pulses at a setting of 40. Aliquots of the cytoplasmic fraction and the nuclear sonicate were counted to determine radioactivity present in these fractions. The rest of the fractions were brought to 10% (w/v) with trichloroacetic acid (TCA). The precipitate after 30 minutes at 0° was washed twice with 5 ml 5% (w/v) TCA and digested with 1.5 ml 0.5 N NaOH at 37° for 90 min. DNA was precipitated by addition of 0.15 ml 70% perchloric acid for 30 min at 0° and hydrolyzed with 3 ml 1.6 N perchloric acid at 70° for 20 min. Aliquots of the resulting supernatant were assayed for radioactivity and for DNA content. Figure 1 shows that after a lag period of 5 minutes incorporation proceeds linearly for at least 40 minutes, and Figure 2 demonstrates that maximal incorporation is achieved with a radioactivity concentration of 5 uCi/ml, corresponding to a thymidine concentration of 0.02 mM.

In vivo. Animals were injected intraperitoneally with a dose of 50 uCi [methyl-^{3}H] thymidine/100 g body weight. Forty minutes later the animals were decapitated and prostates removed, placed on ice, weighed, chopped as above, and washed once with cold PBS. Nuclei were then isolated as described below and processed as for the in vitro assay.

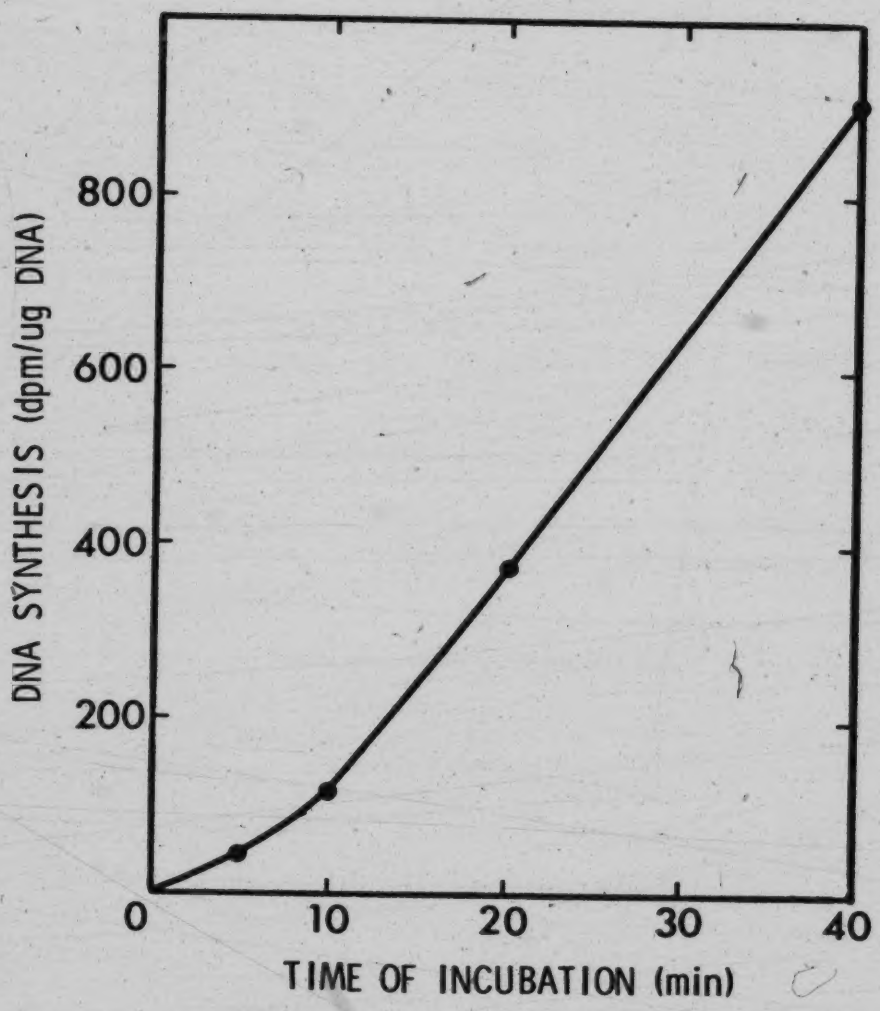

Figure 1. Time course of DNA synthesis *in vitro*. Prostatic minces from 7 day castrates treated for 2 days with 400 ug dihydrotestosterone/100 g body weight (see Chapter IV) were incubated with 5 uCi [methyl-^{3}H] thymidine/ml. After various times of incubation, incorporation of label into DNA was determined. Each value is the mean of two separate determinations.

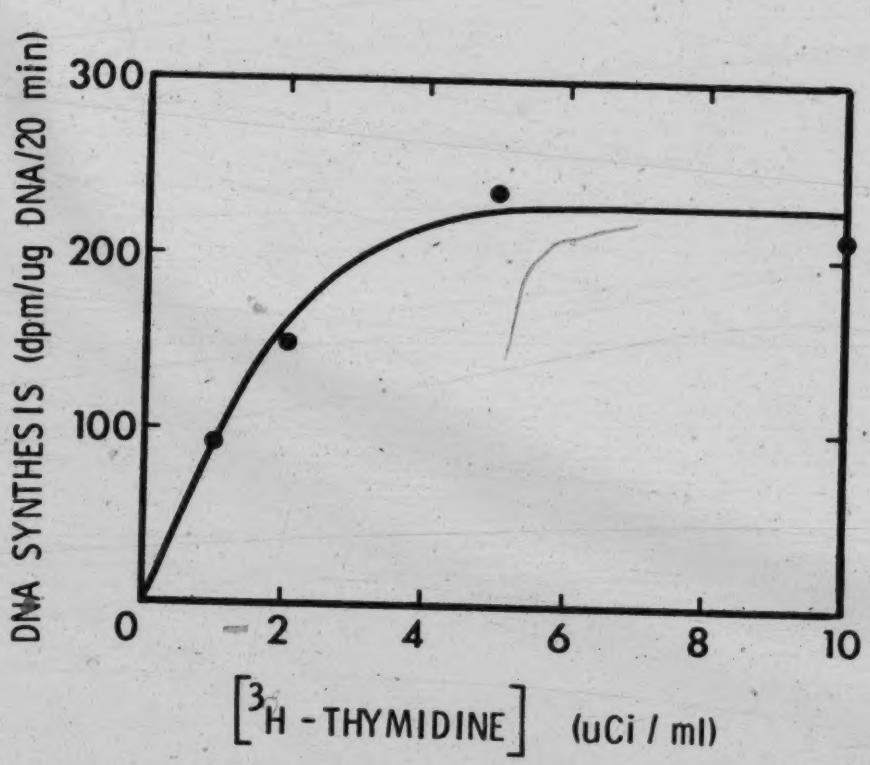

Figure 2. Effect of concentration of thymidine on DNA synthesis *in vitro*. Prostatic minces from 7 day castrates treated for 2 days with 400 ug dihydrotestosterone/100 g body weight (see Chapter IV) were incubated for 20 minutes with varying amounts of [methyl-^{3}H] thymidine at a constant specific activity of 50 Ci/mmole and incorporation of label into DNA determined. Each value is the mean of two separate determinations.

ISOLATION OF NUCLEI

All operations were carried out at 0-2°. The chopped, washed prostatic tissue was suspended in about 20 ml of Tris buffer containing 0.25 M sucrose and 1.5 mM calcium chloride, and manually homogenized in a Dounce homogenizer (Kontes Glass Co., Vineland, N.J.) with 25 strokes of the loose fitting pestle. The suspension was filtered through three layers of gauze, homogenized with 10 strokes of the tight fitting pestle, and centrifuged at 700xg for 10 min. The supernatant was decanted and processed as the cytoplasmic fraction.

Two alternative procedures were followed for processing the nuclear pellet, yielding either "crude" or "Triton-purified" nuclei. In the first case the nuclear pellet was washed in Tris buffer containing 0.05 M sodium chloride and 1.5 mM calcium chloride and resuspended in 5 ml of the same solution. The suspension was centrifuged at 40xg for 2 min to remove debris, and the supernatant decanted as the "crude" nuclear fraction. Nuclei prepared by this method showed cytoplasmic adhesions under light microscope examination and considerable debris was generally present. This procedure yielded about 10^8 nuclei per g tissue and was used in experiments where cytoplasmic contamination was not important. Unless specified otherwise this was the nuclear isolation procedure employed. In cases where it was essential to have clean nuclei (Chapter V) the nuclear

pellet was washed with about 7 ml PBS containing 0.05% (w/v) octylphenoxy polyoxyethanol (Triton X-100; Sigma) and resuspended in PBS. The suspension was centrifuged at 40xg for 2 min to remove debris and the supernatant decanted as the "Triton-purified" nuclear fraction. Light microscope examination revealed no visible cytoplasmic tags and very little debris. This procedure yielded 1.5-2x10^8 nuclei per gram tissue.

To determine nuclear yields, an aliquot of the suspension was stained with methylene blue and the number of nuclei counted using a Spencer Bright-Line Hemacytometer (American Optical Corp., Buffalo, N.Y.).

In all experiments it is assumed that nuclear yields are constant and that the isolated nuclei represent a random sampling of prostatic nuclei.

AUTORADIOGRAPHY

Aliquots of nuclear suspension were centrifuged at 700xg for 5 min. The pellets were resuspended in 1 ml 10% (v/v) PBS in water for 5 min to swell nuclei and then fixed twice in 1 ml acetic acid/methanol (3:1, v/v). After fixation the nuclei were resuspended in 0.2 ml acetic acid/methanol, applied to microscope slides, and air dried. The slides were dipped in Ilford L4 emulsion (Ilford, England), exposed at 0° in total darkness for 2-6 weeks, developed in Kodak D19

developer (Canadian Kodak Sales, Toronto, Ont.) and stained with hematoxylin and eosin.

Alternatively, prostates were dehydrated, embedded in paraffin, and sectioned at 4 um thickness. The slices were applied to slides, rehydrated, and dipped in Kodak NTB2 emulsion. After 4-6 weeks exposure the slides were developed in Kodak Dektol and stained with hematoxylin and eosin.

For determination of percentages of labeled nuclei, 500 or 1000 nuclei were counted at random three times and the results averaged.

III. ATROPHY OF THE PROSTATE FOLLOWING CASTRATION

INTRODUCTION

Moore et al (1930) studied the cytologic changes occurring in the adult rat prostate following castration using the light microscope and found that within four days cell height is reduced, cytoplasmic structure begins to break down, and nuclei become small and pyknotic. These changes are confined mainly to epithelial elements (MacKenzie et al, 1963) with the result that the interglandular stroma becomes relatively more prominent (Woodruff and Perez-Mesa, 1962). With the electron microscope, Harkin (1957) showed that within 24 hours the apical cisternae of the rough endoplasmic reticulum dilate. This is followed by their gradual collapse and depletion of ribosomes (Brandes and Groth, 1963), coinciding with suppression of prostatic secretory activity. At the same time, large numbers of lysosomes appear (Brandes, 1966; Harkin, 1957; Harkin, 1963) and their hydrolytic enzymes are believed to be responsible for degradation and removal of cellular material (De Duve, 1959).

A reduction in concentration of biochemical constituents of prostatic tissue and secretions, e.g., fructose, citrate and acid phosphatase, is among the most

sensitive indicators of the prostatic response to castration (Brandes and Bourne, 1963; Huggins, 1947; Kirchheim and Scott, 1965; Mann, 1964). The activity of several enzymes involved in prostatic secretory function decreases following castration (Singhal and Valadares, 1968). This is probably due to a reduction of prostatic RNA polymerase activity, resulting in a deficiency of ribosomal and messenger RNA which in turn leads to impaired protein synthesis. After 2 days, less than 40% of this capacity remains (Brandes and Bourne, 1963; Mangan et al, 1967), and by 4 days the yield of prostatic ribosomes is reduced by 70% (Mangan et al, 1967; Butler and Schade, 1958; Williams-Ashman et al, 1964). As expected, lysosomal hydrolytic enzymes such as acid phosphatase, aminopeptidase and esterases increase in activity (Brandes, 1966). The respiration rate, oxygen consumption and rate of respiration-coupled synthetic activities of rat prostatic tissue decline following castration (Butler and Schade, 1958; Nyden and Williams-Ashman, 1953) due to loss of mitochondria (Edelman et al, 1963; Pegg and Williams-Ashman, 1968). Anaerobic glycolysis, however, does not appear to be affected (Barron and Huggins, 1944; Butler and Schade, 1958; Nyden and Williams-Ashman, 1953; Price and Williams-Ashman, 1961). Because of loss of prostatic secretion and a relative decrease in cytoplasmic volume of epithelial cells, the DNA concentration increases (Kochakian, 1963; Lostroh, 1962; Williams-Ashman et al, 1964), although the actual amount of DNA per prostate

decreases.

In order to establish for regeneration studies (Chapter IV) the appropriate length of time between castration and initiation of hormone treatment, the precise time course of atrophy of the rat ventral prostate was determined. The parameters of wet weight and nuclear content were measured as indicators of prostatic function, and rate of DNA synthesis and changes in nuclear content were used as indices of proliferative activity. In addition, studies were performed to test whether loss of cells from the prostate following castration is a random process or whether a particular subpopulation in the normal prostate might be predestined to survive castration.

RESULTS

Decline of DNA synthesis, wet weight, and nuclear content of the prostate following castration. At various times after castration, prostates were removed and weighed. The rate of DNA synthesis was assayed *in vitro* and nuclei were isolated and counted as described in Chapter II. Figure 3 shows that the immediate effect of castration is a decline in rate of incorporation of thymidine to 25% of normal by day 1 and to less than 5% of normal by day 6, indicating that what little proliferation there is in the normal prostate stops rapidly. Both prostatic weight and nuclear content remain within the normal range until day 4, and then

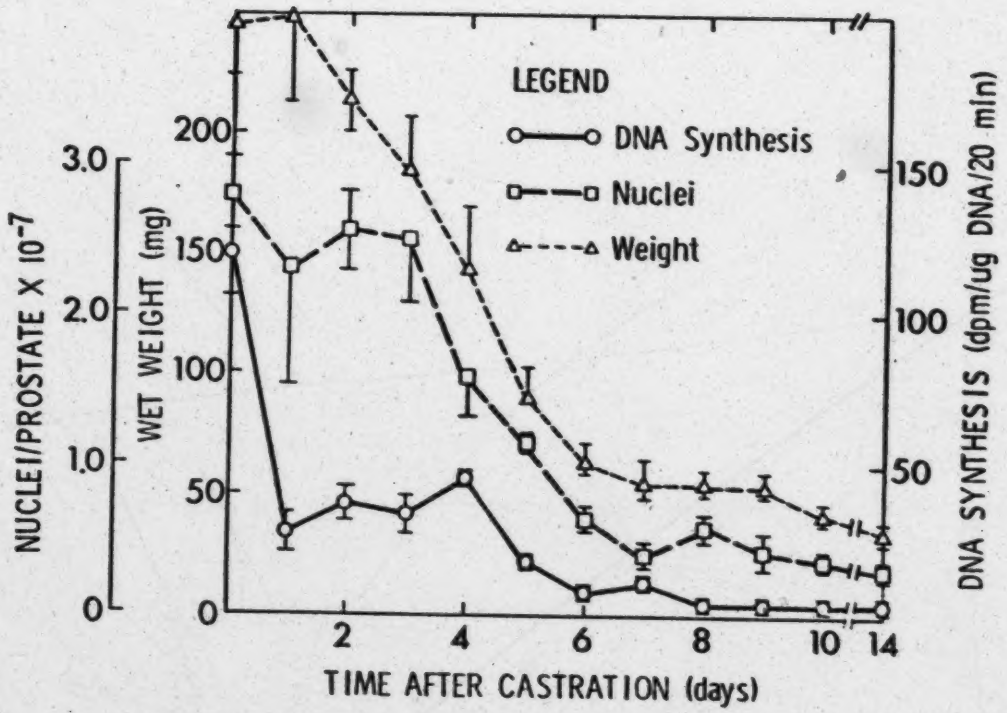

Figure 3. Effects of castration on the prostate. Animals were castrated at day 0. At various times thereafter groups of rats were killed and prostates processed as in Chapter II. Values for normal animals are shown on the ordinate. Each point represents the mean ± S.E. for at least three experiments.

decrease to about 20% and 15% of normal, respectively, by day 7, after which time very little further change occurs. The decline in nuclear content and weight occur at the same rate, so that DNA concentration remains constant.

Light microscope examination of prostate slices indicates that, as discussed in the Introduction, the main cytological effects of castration are greatly reduced epithelial height, decreased acinar size, and increased amounts of connective tissue between acini.

Cell survival following castration. In the normal prostate, there is a moderate rate of DNA synthesis, presumably to replace cells lost due to functional turnover. To test the possibility that these proliferating cells might preferentially survive castration, normal animals were continuously labeled with ^{3}H-thymidine for 72 hours. 32 hours after the last injection, by which time unincorporated label should have been eliminated, animals were castrated. At various times after castration animals were killed and both the amount of label present in the prostate and the labeling index (i.e., the percentage of labeled nuclei) determined. Figure 4 shows that both parameters remain essentially constant during prostatic atrophy, indicating no selective retention of labeled cells.

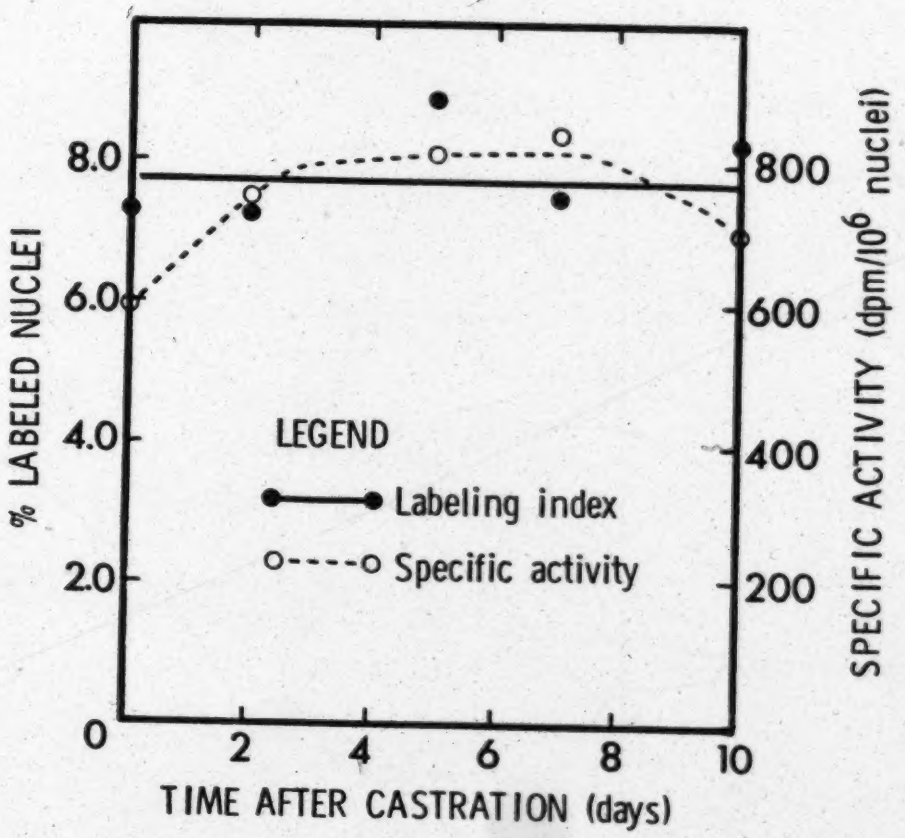

Figure 4. Survival of labeled cells following castration. Normal rats were injected intraperitoneally every 8 hours with 25 uCi [methyl-^{3}H] thymidine/100 g body weight for a total of 9 injections. 32 hours after the last injection, the animals were castrated. At various times after castration groups of rats were killed and prostatic nuclei isolated. Nuclear radioactivity was determined by scintillation counting and the labeling index by autoradiography.

DISCUSSION

The above results suggest that the process of prostatic atrophy following castration can be divided into three distinct stages. Firstly, from days 1-3 following castration, the rate of DNA synthesis has fallen dramatically from the normal rate but wet weight and nuclear content remain in the normal range. Secondly, during days 3-6, wet weight and nuclear number decrease rapidly. Finally, after day 6, all three of these parameters are maintained at approximately the same reduced level. In the present study, prostatic weight decreased to 36 mg by 14 days after castration, the longest time studied. Burkhart (1942) has reported that after 40 days, prostatic weight decreases further to 14 mg (correcting for body weight of animals), a rate of about 3% per day, as compared to 25-30% per day during the period of rapid atrophy from days 3-6.

The interpretation of the studies on cell survival is complicated by the possibility that proliferating cells in the normal prostate might produce labeled daughter cells that entered the population of differentiated cells and hence would not preferentially survive castration. The loss of these labeled differentiated cells might counteract any tendency for selective retention of labeled proliferating cells to increase the labeling index. However, the finding that the labeling index remains constant at 8% throughout the period of prostatic atrophy following castration

indicates that overall loss of labeled cells is random. Hence, selective retention of labeled cells in the proliferating compartment would have to be accompanied by selective loss of labeled cells from the differentiated compartment in order for the labeling index to remain constant; this possibility seems very unlikely. Therefore it appears that if there is a subpopulation of cells in the normal prostate that preferentially survives castration, it cannot be distinguished on the basis of its ability to incorporate labeled thymidine. This does not eliminate the existence of such a subpopulation which might be distinguished in other ways. However the lack of methods of permanently labeling or distinguishing cells other than labeled thymidine would appear to preclude further studies along these lines.

In summary, the time course of atrophy of the rat prostate has been characterized in detail, and three stages in the atrophic process have been identified - shutdown of DNA synthesis, followed by rapid loss of cells, and then a period during which a reduced prostatic size is maintained. Based on ability to incorporate labeled thymidine, there does not appear to be a subpopulation in the normal prostate that preferentially survives castration.

IV. EFFECTS OF ANDROGENS ON DNA SYNTHESIS AND CELL DIVISION IN THE CASTRATE RAT PROSTATE

INTRODUCTION

Androgens can reverse all the cytological and biochemical changes that occur in the prostate following castration (Moore et al, 1930; Price and Williams-Ashman, 1961). In fact, the response of sex accessory organs in castrate animals was used as a bioassay during the purification of androgens from testicular extracts (Moore et al, 1930).

Burkhart (1942) performed the first direct study of the proliferative effects of androgens on the sex accessory organs of castrate rats. She injected various doses of testosterone propionate at daily intervals and measured the mitotic activity in prostate and seminal vesicle slices using colchicine to arrest cells in mitosis. In 40 day castrates a wave of mitotic activity occurred, beginning around 36 hours, peaking at about 48 hours, and ending around 96 hours after initiation of treatment. In shorter term castrates (10 and 20 days) the response was essentially the same. The time of the wave of mitotic activity depended to a small extent on the dose of hormone administered, occurring earlier with larger doses. The findings of

Burkhart have been confirmed in mouse prostate by Allen (1958).

The first report of stimulatory effects of androgens on incorporation of ^{3}H-thymidine by prostates from castrate rats was made by Sheppard et al (1965). Increased incorporation was observed after 2 days of treatment but not after 7 days. Williams-Ashman and coworkers (Kosto et al, 1967; Coffey et al, 1968) performed more detailed studies of the effects of testosterone propionate in 7 day castrate rats on incorporation of ^{14}C-thymidine into DNA *in vivo* and by prostatic slices *in vitro*, and on the activity of DNA polymerase. DNA polymerase activity required Mg^{++}, denatured DNA template, ATP, and all four deoxynucleoside triphosphates. They found no effects after 24 hours, but a massive stimulation of all activities by 48 hours. Incorporation of thymidine peaked at 72 hours and DNA polymerase activity reached a maximum at 96 hours, and then all activities decreased rapidly despite continued daily administration of hormone, so that by 10 days after initiation of treatment they had returned to the normal levels. Hormone treatment stimulated increases in prostatic weight and in DNA, RNA and protein content. Maximal increase in DNA content occurred within two weeks and could not be increased further by treatment for as long as 25 days, but prostatic weight continued to increase up to 25 days. Some of these findings have been reproduced in rats by Doeg et al

(1972) and in hamsters by Giegel et al (1971).

Bruchovsky and Wilson (1968a) and Anderson and Liao (1968) reported that dihydrotestosterone is selectively concentrated by rat prostatic nuclei after an intravenous injection of labeled testosterone. The enzyme responsible for the conversion is 5alpha-reductase (Bruchovsky and Wilson, 1968a; Wilson and Gloyna, 1970). The cytoplasm contains many metabolites of androgens and dihydrotestosterone comprises only 5% of cytoplasmic androgens, but 75% of the steroid in nuclei is dihydrotestosterone, with most of the remainder testosterone. Much of the intracellular steroid is found associated with specific protein receptors (Bruchovsky and Wilson, 1968b; Unhjem et al, 1969; Fang et al, 1969; Mainwaring, 1969). The functional significance of these receptors is unclear, but it seems likely that they may be involved in the selective uptake of dihydrotestosterone by prostatic nuclei (Pennie and Bruchovsky, 1973). In prostates in organ culture (Baulieu et al, 1968; Robel et al, 1971) testosterone is more active in maintaining epithelial height and stimulating secretion, while dihydrotestosterone has greater effects on epithelial hyperplasia. In vivo Schmidt et al (1972) found that dihydrotestosterone produces a greater increase in DNA content of prostates of immature castrate rats than testosterone, and Tuohimaa et al (1973) observed a slightly more rapid and extensive increase of labeling index in the prostates of one month castrate

adult rats in response to dihydrotestosterone as compared to testosterone. These findings indicate that the conversion of testosterone to dihydrotestosterone may be important in the proliferative effects of androgens on target tissues. Of interest in this regard is the report by Siiteri and Wilson (1970) and confirmation by Giorgi et al (1971, 1972) of increased uptake and retention of dihydrotestosterone in prostates of patients with benign prostatic hyperplasia as compared to normal prostates.

As has been discussed in Chapter I, the growth and function of the prostate can be inhibited by natural and synthetic estrogens. However because of their biological potency these compounds have many properties besides their antiandrogenic activity that lead to undesirable side effects. The search for antiandrogens that lack these side effects has resulted in two compounds that appear to be promising - the steroid cyproterone acetate and the non-steroid SCH 13521. Both drugs antagonize the effects of androgens on the growth and function of the castrate rat prostate (Geller et al, 1969; Walsh and Korenman, 1970; Neri et al, 1972). Cyproterone acetate appears to compete with dihydrotestosterone for uptake into prostatic nuclei (Fang et al, 1969; Fang and Liao, 1969; Walsh and Korenman, 1970; Rennie and Bruchovsky, 1972). The mechanism of action of SCH 13521 is uncertain.

The proliferative effects of androgens on the prostate

are always preceded by a period of 1-2 days during which DNA synthesis does not occur but the tissue is very active biochemically. The earliest effect of androgens appears to be actinomycin D sensitive stimulation of prostatic RNA synthesis (Liao et al, 1965; Fujii and Villee, 1968). This RNA is mostly ribosomal RNA (Liao et al, 1966; Liao and Lin, 1967). It has been shown that this stimulation of RNA synthesis is due partly to activation of the template activity of prostatic chromatin (Liao and Lin, 1967; Mangan et al, 1968), and partly to the increased activity of nucleolar RNA polymerase (Williams-Ashman et al, 1964; Mainwaring et al, 1971; Davies et al, 1972). In addition there is indirect evidence for androgenic stimulation of messenger RNA synthesis, based on the ability of prostatic polysomes to incorporate amino acids into proteins (Liao and Williams-Ashman, 1962; Mangan et al, 1967). The accumulation of androgen-induced messenger and ribosomal RNA in the cytoplasm results in increased incorporation of amino acids into proteins (Williams-Ashman et al, 1964; Liao and Williams-Ashman, 1962; Chung and Coffey, 1971; Mainwaring and Wilce, 1972). There also occurs an increase in respiratory metabolism due to increased numbers of mitochondria (Edelman et al, 1963; Doeg, 1968; Pegg and Williams-Ashman, 1968).

In interpreting studies of early androgenic effects it must be borne in mind that, as will be shown in this

chapter, the response of the prostate to hormonal stimulation depends on the duration of castration prior to beginning of treatment. In short term castrates, proliferation does not occur, while in animals castrated for long periods of time androgen does induce a proliferative response. In the former case, RNA and protein synthesis is probably involved in the secretory function of the prostate; in the latter case, these syntheses likely occur in preparation for proliferation. In previous work on the quantitative aspects of stimulation of RNA and protein synthesis these differences do not seem to be very important, but when analysis is concentrated on particular species of RNA or protein, they undoubtedly will.

MATERIALS AND METHODS

Cyproterone acetate (1,2alpha-methylene-6-chloro pregna-4,6-diene-17alpha-ol-3,20-dione-17alpha-acetate) and SCH 13521 (4'-nitro-3'-trifluoromethylisobutyranilide) were gifts of Schering Corp. (Bloomfield, N.J.). Both compounds were injected using the same vehicle and in the same manner as described in Chapter II for hormone administration.

RESULTS

Stimulation by dihydrotestosterone of proliferative and secretory activity of the castrate rat prostate. In order to characterize the various responses of prostatic tissue to

restoration of androgen, animals which had been castrated for varying periods of time were treated with 400 ug dihydrotestosterone/100 g body weight every 24 hours. As will be shown below, this dose of hormone produces maximal effects on incorporation of thymidine. As in Chapter III, the parameters used as a measure of proliferation and functional status are wet weight, number of nuclei per prostate, and incorporation of labeled thymidine into nuclear DNA. Control animals treated with the injection vehicle in the same way as experimental animals showed no differences from untreated castrates (Chapter III), and in no case did significant amounts of acid insoluble material appear in the cytoplasmic fraction.

Normal rats were castrated, and treatment with 400 ug dihydrotestosterone/100 g body weight begun immediately. Figure 5 shows that DNA synthesis is not stimulated, and in fact may be slightly inhibited. There appears to be a slight increase in numbers of nuclei, but even by 4 days the difference from normal is not significant ($P>0.05$). The only significant stimulation seen in 0 day castrates is a marked increase in wet weight at a rate of approximately 20% per day. By 2 days after beginning of treatment the weight is significantly elevated above normal ($P<0.01$).

Similar results are seen when 1 day castrates are treated with daily doses of dihydrotestosterone, as shown in Figure 6. The number of nuclei remains within the normal

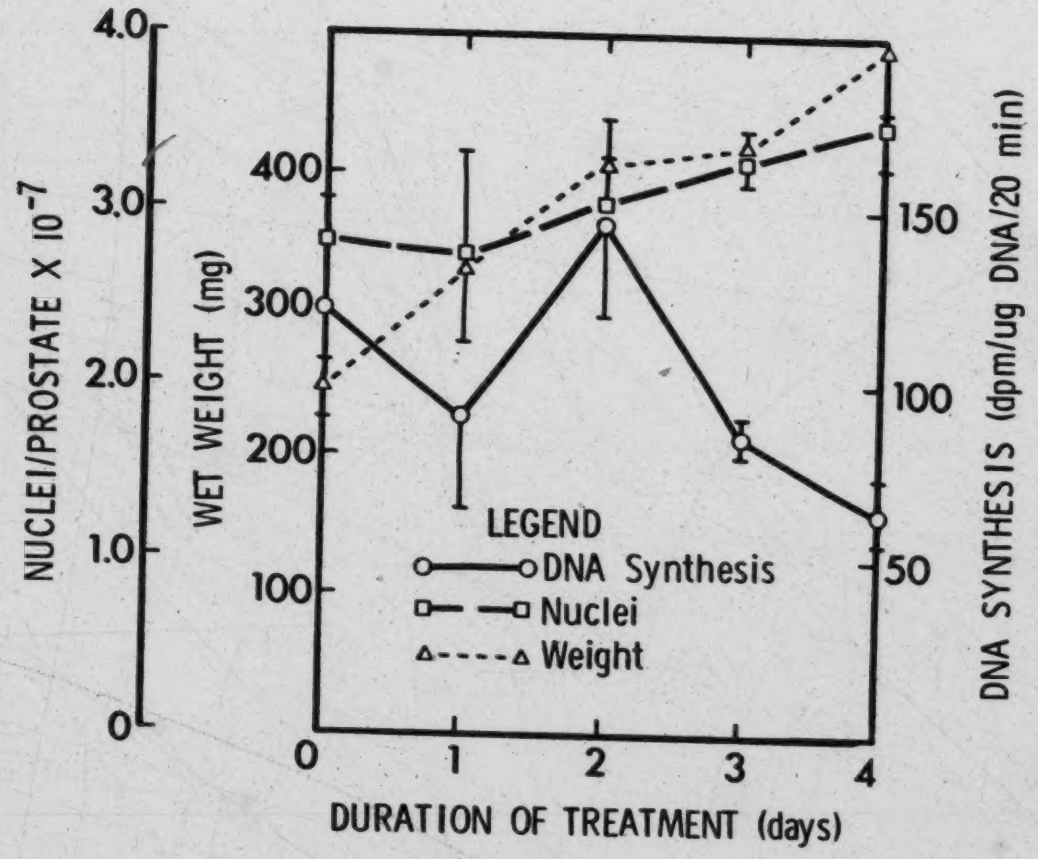

Figure 5. Response of 0 day castrates to dihydrotestosterone. Hormone in a dose of 400 ug/100 g body weight was administered daily as described in Chapter II. After various intervals of treatment, animals were killed and prostates removed and weighed. Rate of incorporation of thymidine was assayed *in vitro* as described in Chapter II. Each point is the mean±S.E. for 3 separate experiments.

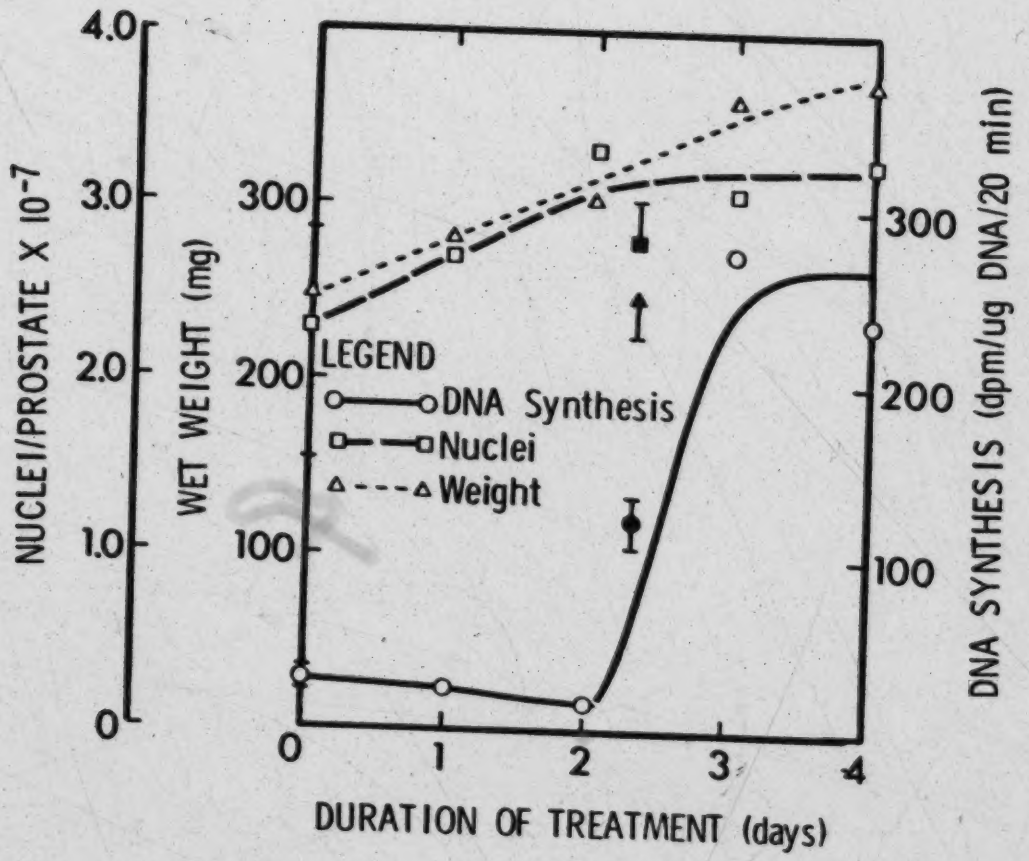

Figure 6. Response of 1 day castrates to dihydrotestosterone. After various intervals of treatment with 400 ug dihydrotestosterone/100 g body weight, animals were killed and prostates removed and weighed. Rate of incorporation of thymidine was assayed *in vitro* as described in Chapter II. Values for 1 day castrates are shown on the ordinate. The closed symbols represent the corresponding values for normal rats. Error bars, where indicated, represent the mean±S.E. for three separate experiments; otherwise each point represents a single experiment.

range, while prostatic weight increases significantly. Incorporation of thymidine remains low for 2 days, then increases to a level near the normal rate of incorporation. The values are about twice normal, but this may be due to experimental variation.

The results obtained when 4 day castrates, whose prostates are in the process of atrophy, are treated with daily doses of 400 ug dihydrotestosterone/100 g body weight are shown in Figure 7. The process of atrophy continues for 1 day following initiation of treatment, then DNA synthesis is stimulated and the number of nuclei starts to increase. Rate of DNA synthesis reaches a maximum at day 4 and then declines, and the number of nuclei has returned to normal by day 5. After this time the greatly reduced rate of DNA synthesis and of production of nuclei indicate that proliferation has essentially stopped. The wet weight, on the other hand, continues to increase beyond its normal level and by day 7 is 70% higher than normal.

Figure 8 shows the response of 7 day castrates to daily administration of 400 ug dihydrotestosterone/100 g body weight. There is a lag period of 24-36 hours after beginning of treatment before incorporation of labeled thymidine into nuclear DNA increases rapidly to a maximal rate by the third day of about 200 times the rate for untreated controls and 10 times the normal rate. These increased rates of incorporation are due firstly to a greater uptake of labeled

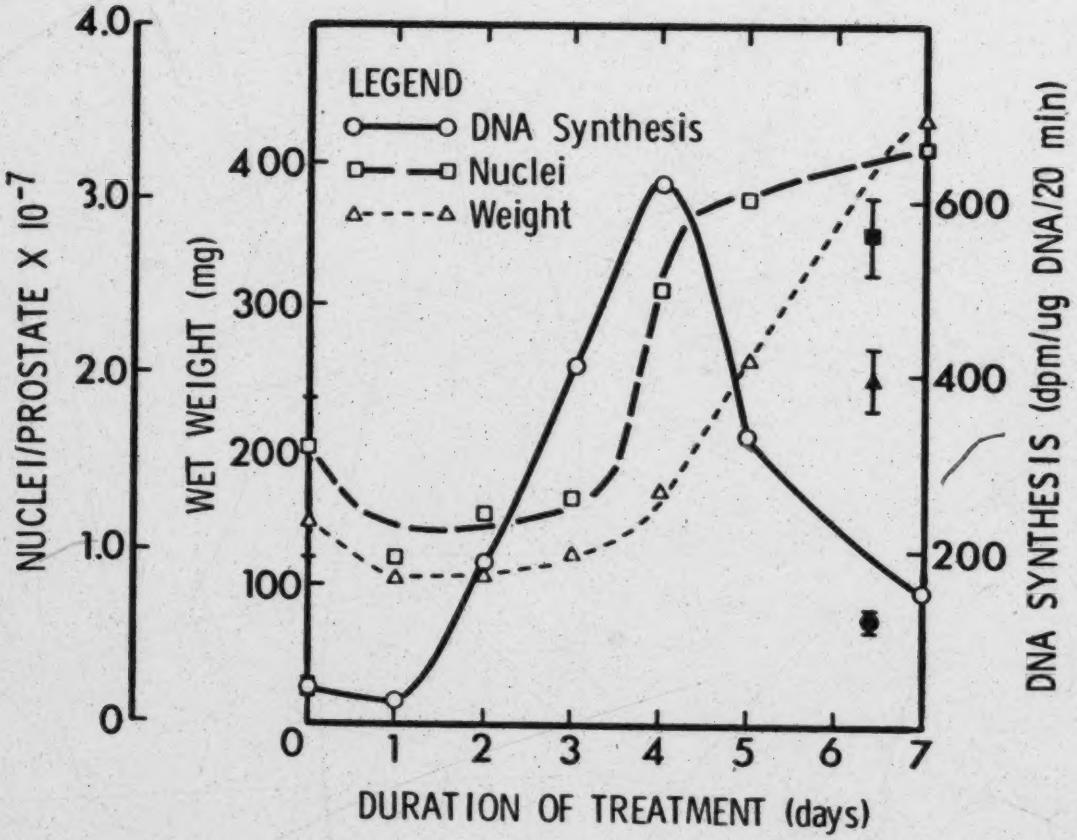

Figure 7. Response of 4 day castrates to dihydrotestosterone. After various intervals of treatment with 400 ug dihydrotestosterone/100 g body weight, rats were killed and prostates removed and weighed. Rate of incorporation of thymidine into DNA was assayed in vitro as described in Chapter II. Values for 4 day castrates are shown on the ordinate. The closed symbols represent the corresponding values for normal rats. Error bars, where indicated, represent the mean±S.E. for three separate experiments; otherwise each point represents a single experiment.

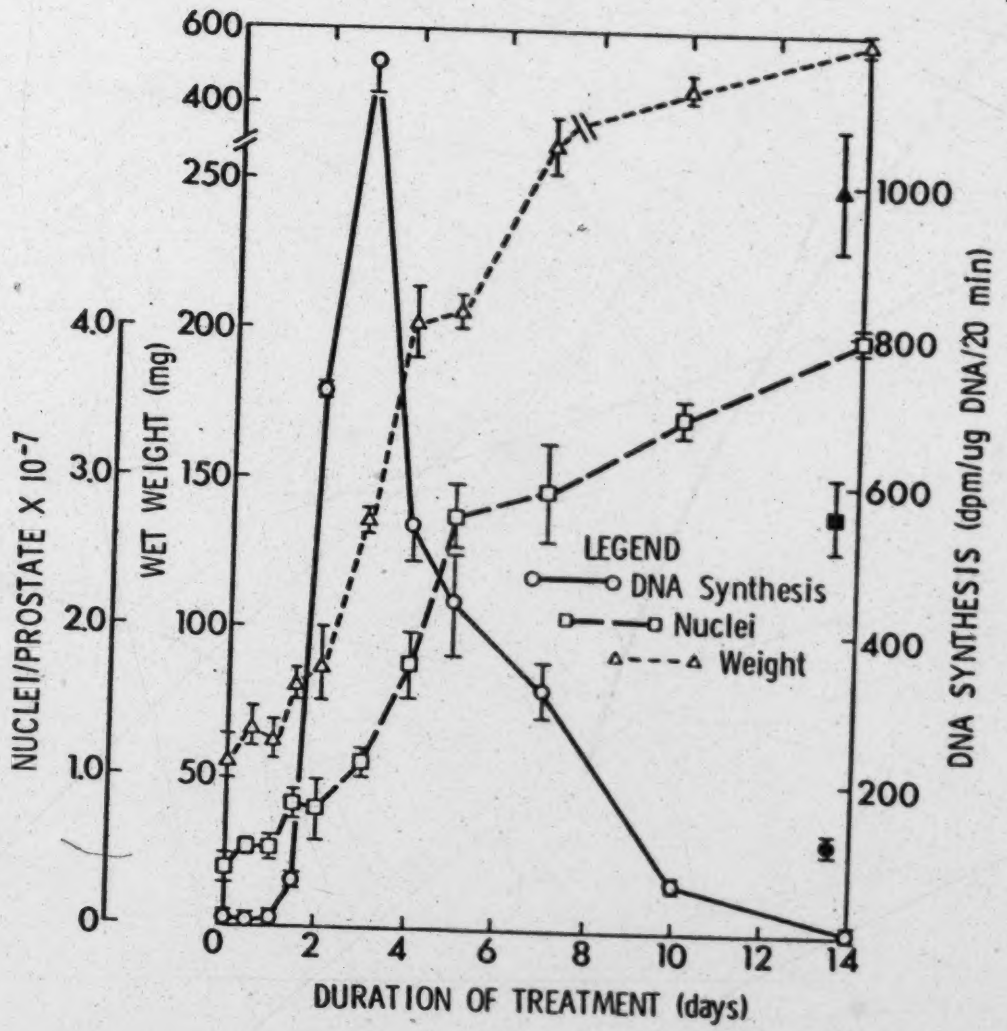

Figure 8. Response of 7 day castrates to dihydrotestosterone. After various periods of treatment with 400 ug dihydrotestosterone/100 g body weight, rats were killed and prostates removed and weighed. Rate of incorporation of thymidine into DNA was assayed *in vitro* as described in Chapter II. Values for 7 day castrates are shown on the ordinate. The closed symbols represent corresponding values for normal rats. All values are the mean±S.E. for at least three separate experiments.

thymidine by nuclei, and secondly to an increase in the percentage of nuclear label converted into acid-insoluble material from less than 10% in controls to 100% in hormone treated tissue. After the third day, despite continued administration of hormone, the rate of incorporation declines. By day 10 it has dropped to below the normal level and by day 14 to the level observed in control untreated castrates. The percentage of labeled nuclei as determined by autoradiography correlates directly with levels of incorporation of thymidine (correlation coefficient = 0.78). Thus changes in rate of incorporation are due to participation by varying numbers of nuclei rather than variations in rate of synthesis among individual nuclei. At the peak of incorporation at day 3, about 3% of nuclei are labeled in vitro . Wet weights and numbers of nuclei per prostate are significantly above the control level ($P<0.01$) by 36 hours after beginning of treatment, and increase rapidly between day 2 and day 5. During this time the doubling time for the cell population is about 40 hours and the weight increases at a rate of about 40-50% per day. By day 5 and day 7 the number of nuclei and wet weight, respectively, have returned to the normal level. After this time the rate of increase in numbers of nuclei decreases to 25% of the former rate, while weights continue to increase linearly for at least 14 days (note change of scale in Figure 8). By day 14, weights are twice the normal level while nuclear number is about 30% greater than normal. The

continued slow increase in number of nuclei after day 5 may represent an overshoot due to the high doses of hormone administered.

In order to ascertain that the effects on incorporation of thymidine by minces *in vitro* are not an artefact of the *in vitro* incubation, the rate of incorporation was assayed *in vivo* as described in Chapter II. Figure 9 demonstrates that the time course of incorporation *in vivo* is the same as that observed *in vitro*. When the nuclei were examined by autoradiography it was found that, as in the *in vitro* assay, there is excellent correlation (correlation coefficient = 0.99) between rate of incorporation of thymidine and the percentage of labeled nuclei. At day 3, the peak of incorporation, 13% of the nuclei are labeled.

Examination of prostate slices under the light microscope reveals that hormone induced regeneration results in restoration of the normal morphology, with decreased connective tissue, large acini, and tall columnar epithelium.

In order to determine the length of time a dose of androgen remains effective, prostates were examined at various intervals after administration of a single dose of 400 ug dihydrotestosterone/100 g body weight to 7 day castrates. The results are shown in Figure 10. It is evident that a single dose of hormone can induce an increased rate

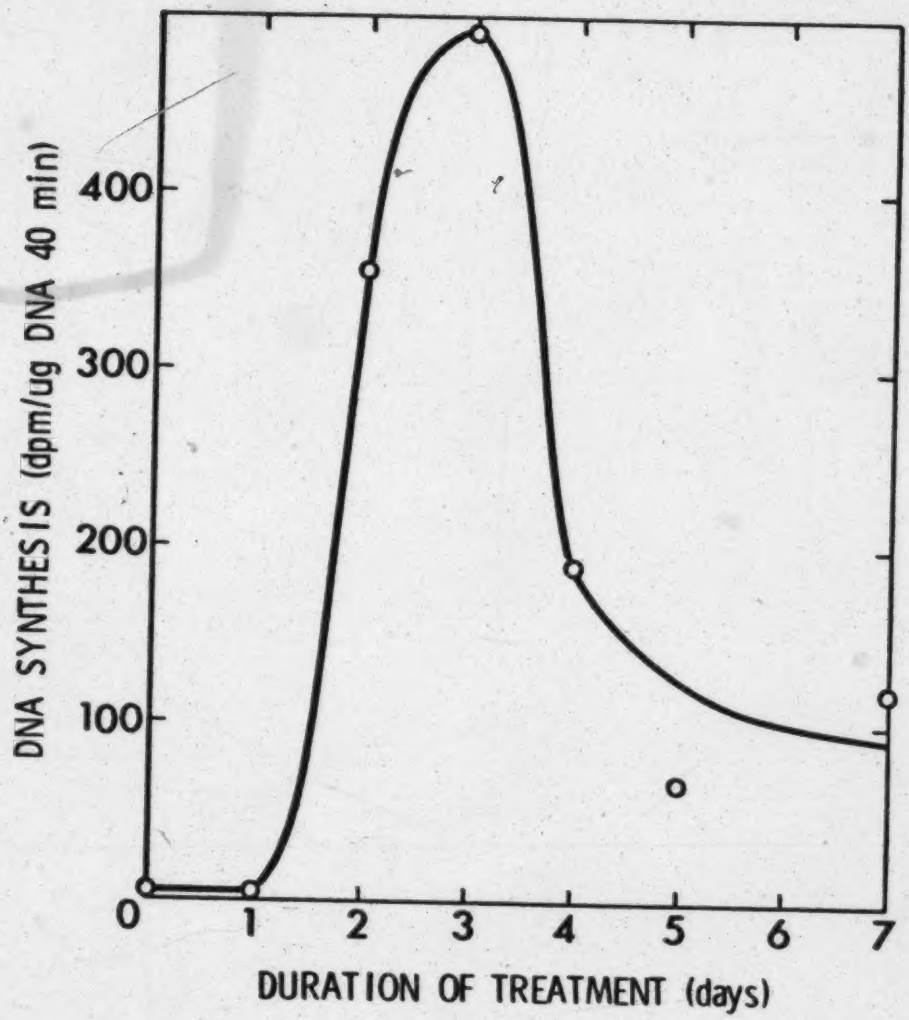

Figure 9. Incorporation of thymidine *in vivo*. Seven day castrates were treated with daily doses of 400 ug dihydrotestosterone/100 g body weight and rate of incorporation of thymidine *in vivo* assayed after various periods of treatment. Each point represents a single experiment.

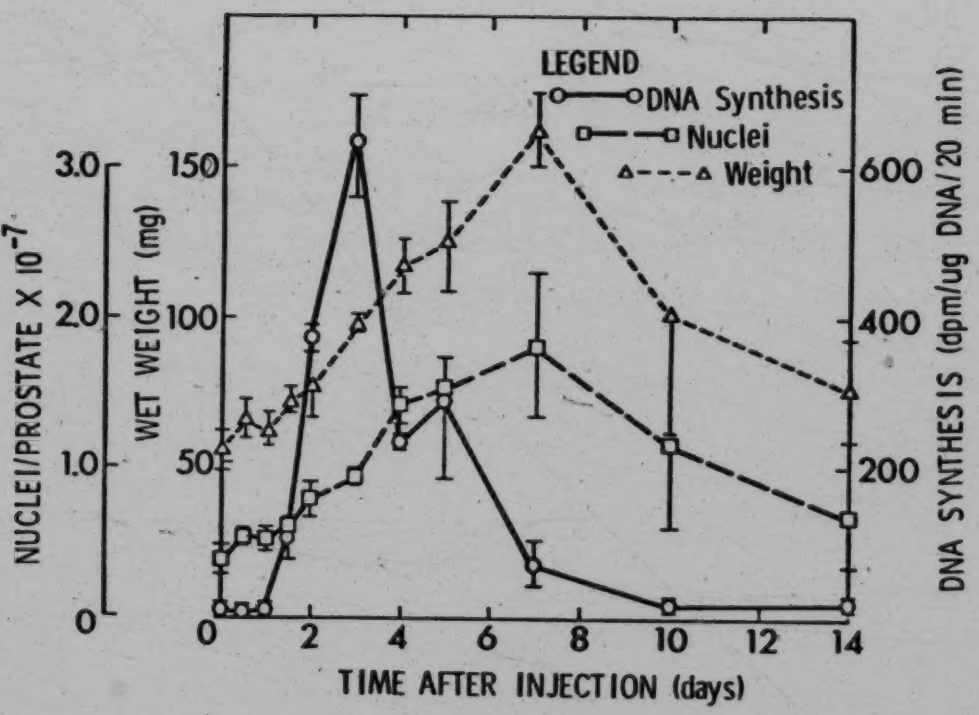

Figure 10. Response of 7 day castrates to a single dose of dihydrotestosterone. At various times after a single injection of 400 ug dihydrotestosterone/100 g body weight, rats were killed and prostates removed. Incorporation of thymidine was assayed in vitro as described in Chapter II. Each point represents the mean±S.E. for at least 3 separate experiments.

of DNA synthesis with the same time course, although a smaller magnitude, as observed during daily administration. Both prostatic weight and nuclear numbers are stimulated to increase for 7 days at about half the rate produced by multiple doses of hormone, but neither attains the normal level. It is uncertain whether hormone is actually present in the prostate throughout the time growth is stimulated by a single dose, but the rapid metabolism and excretion of androgens compared to the length of time that effects are observed raises the possibility that continued presence of hormone may not be necessary for maintenance of proliferation once it has begun.

Effects of testosterone on prostatic regeneration. In order to compare the relative potencies of testosterone and dihydrotestosterone, 7 day castrate rats were treated with daily doses of 400 ug testosterone/100 g body weight. Figure 11 shows that the DNA synthetic response induced by testosterone is very broad and variable, unlike the sharp peak observed with dihydrotestosterone treatment. Furthermore, the rates of increase of wet weight and nuclear content are only about 25% of those elicited by dihydrotestosterone, and by day 3 both parameters are significantly less ($P<0.05$) than those for dihydrotestosterone treated animals. It takes 14 days for testosterone to return wet weight and number of nuclei to their normal values, while dihydrotestosterone does so in 7 and 5 days, respectively.

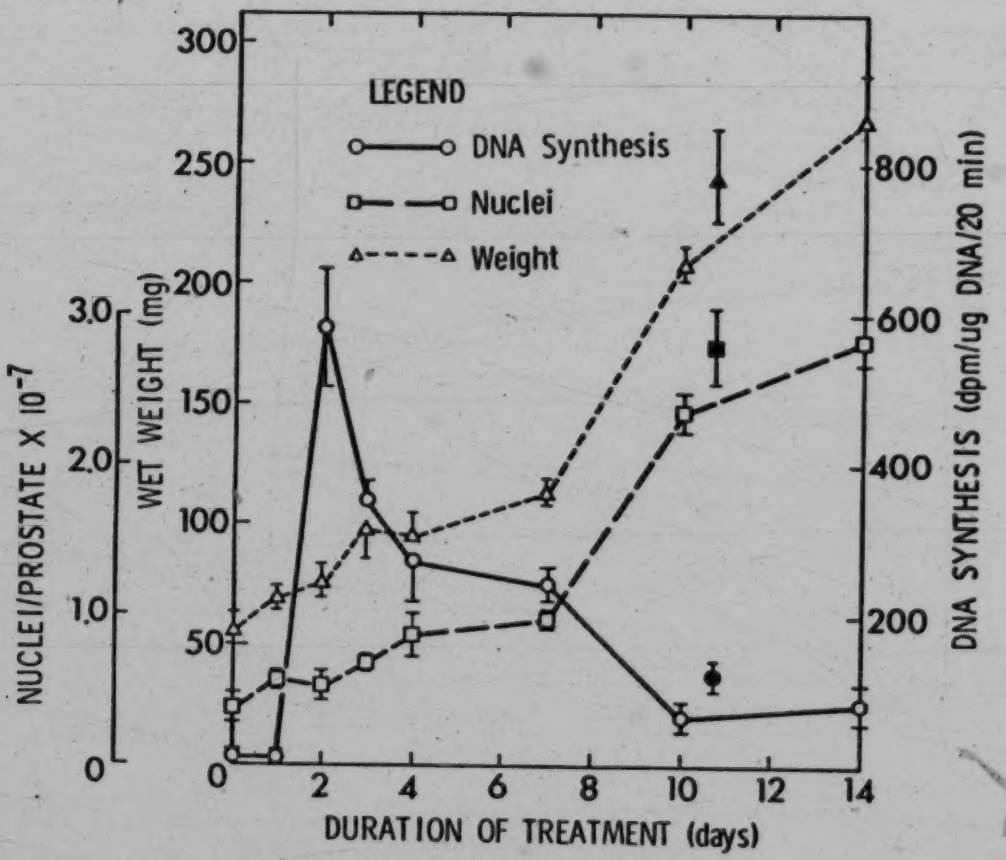

Figure 11. Effect of testosterone on growth of the prostate. After varying periods of treatment of 7 day castrates with daily doses of 400 ug testosterone/100 g body weight, rats were killed and prostates removed. Rate of incorporation of thymidine was assayed *in vitro* as in Chapter II. Values for normal rats are shown as closed symbols. Results are expressed as mean±S.E. for at least three separate experiments.

Effect of dose of androgen on DNA synthesis. In order to determine the relative effects of testosterone and dihydrotestosterone on the initial stimulation of rate of DNA synthesis, rats were treated daily for 2 days with various doses of testosterone or dihydrotestosterone and rate of incorporation of thymidine determined in vitro . As can be seen in Figure 12, at all doses except the lowest the values for dihydrotestosterone are consistently greater than those for testosterone. However, due to considerable variability in the response after this short period of treatment, the differences are statistically significant ($P<0.05$) only at the highest dose tested.

Effect of other hormones on rate of DNA synthesis. The effects of androstanedione, 3alpha-androstanediol, 3beta-androstanediol and 17beta-estradiol on stimulating rates of incorporation of thymidine by 7 day castrates were compared to those of testosterone and dihydrotestosterone. Animals were treated daily for 2 days with either 200 or 400 ug/100 g body weight. Results are shown in Table 1. It is evident that 3alpha-androstanediol is as potent as DHT in stimulating initial rates of DNA synthesis, but none of the other hormones tested has very dramatic effects.

Effect of antiandrogens on DNA synthesis. The effects of the steroidal antiandrogens estradiol and cyproterone acetate and the non-steroidal antiandrogen SCH 13521 on stimulation by testosterone or dihydrotestosterone of DNA

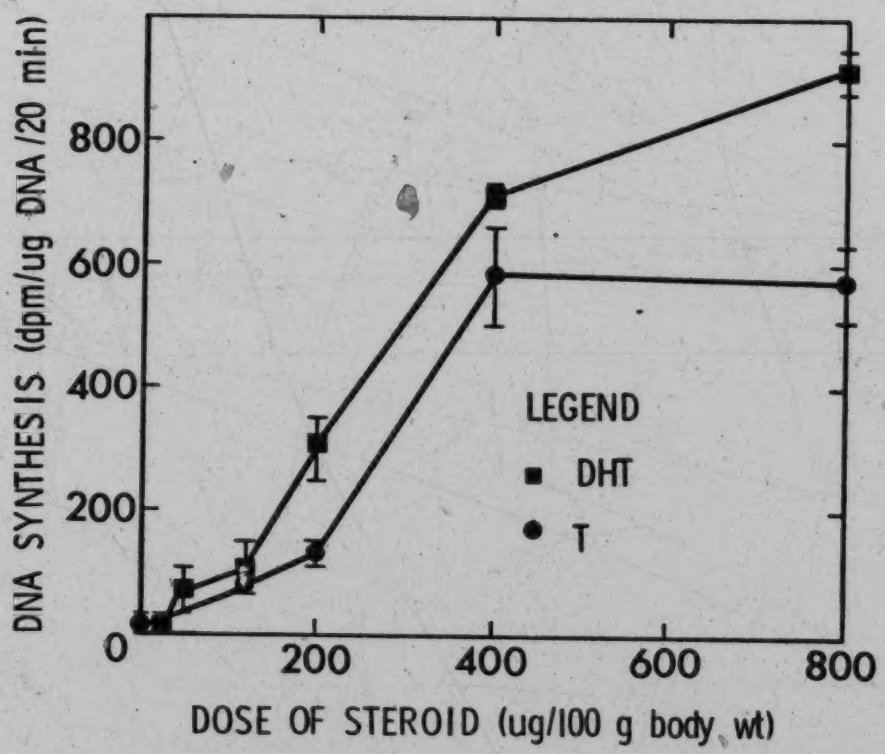

Figure 12. Effect of dose of testosterone or dihydrotestosterone on rate of DNA synthesis. 7 day castrates were injected with various doses of testosterone or dihydrotestosterone. The injection was repeated 24 hours later. 48 hours after the initial injection the rats were killed and rate of DNA synthesis assayed in vitro as in Chapter II. Each value represents the mean±S.E. for at least three separate determinations.

	RADIOACTIVITY INCORPORATED (dpm/ug DNA/20 min)	
HORMONE	200 ug/100g	400 ug/100g
NONE	4.2±0.7	4.2±0.7
DHT	301.9±55.0	716.1±11.5
T	132.9±21.6	582.6±77.4
A'dione	9.6	15.2
3a-A'diol	273.5	–
3b-A'diol	33.5	–
E'diol	–	3.7

Table 1. Effects of various hormones on DNA synthesis. Animals were injected with either 200 or 400 ug/100 g body weight of various hormones. Abbreviations used are DHT for dihydrotestosterone, T for testosterone, A'dione for androstanedione, 3a-A'diol for 3alpha-androstanediol, 3b-A'diol for 3beta-androstanediol, and E'diol for 17beta-estradiol. The injection was repeated 24 hours later. 48 hours after the first injection the animals were killed and DNA synthesis assayed <u>in vitro</u> as described in Chapter II. Results are shown as mean±S.E. where three separate determinations were made, or else as values for single experiments.

synthesis in 7 day castrates were tested. In all experiments, testosterone or dihydrotestosterone was injected in a dose of 400 ug/100 g body weight. Antiandrogens were injected at the same time as androgen at a different site. To test the effects of estradiol, animals were treated with two doses of androgen plus 200 or 400 ug estradiol/100 g body weight at 24 hour intervals and rate of DNA synthesis assayed in vitro 48 hours after the first injection. To test the effects of cyproterone acetate and SCH 13521, rats were injected with a single dose of dihydrotestosterone plus 400 or 2000 ug antiandrogen/100 g body weight, and rate of incorporation of thymidine assayed in vitro 72 hours after injection. Results are shown in Table 2. The effects of estradiol on dihydrotestosterone-stimulated DNA synthesis are extremely variable, ranging from marked inhibition to marked stimulation. The mean values indicate stimulation, but differences are not significant compared to animals treated with dihydrotestosterone alone. Estradiol inhibits very strongly testosterone-stimulated incorporation of thymidine. The reasons for these effects of estradiol are unclear. Both the antiandrogens, cyproterone acetate and SCH 13521 have strongly inhibitory effects on stimulation of DNA synthesis by DHT. SCH 13521 appears to be slightly more potent, producing a 60-70% inhibition as compared to about 40% for cyproterone acetate.

TREATMENT	RADIOACTIVITY INCORPORATED (dpm/ug DNA/20 min)	(% of control)
DHT	715.1±11.5	100
DHT+E'diol(200)	795.5±344.7	111
DHT+E'diol(400)	1087.4±291.1	152
T	582.6±77.8	100
T+E'diol(400)	45.5±13.2	8
DHT	633.9±63.0	100
DHT+CA(400)	372.2	59
DHT+CA(2000)	367.2	58
DHT+SCH(400)	271.5	43
DHT+SCH(2000)	172.6	27

Table 2. Effects of antiandrogens on rates of DNA synthesis. Animals were treated as described in the text. Abbreviations used are DHT for dihydrotestosterone, T for testosterone, E'diol for estradiol, CA for cyproterone acetate, and SCH for SCH 13521. Rate of DNA synthesis was assayed *in vitro* as in Chapter II. Results are shown as mean±S.E. where at least three different experiments were done, or else as the mean of two values.

DISCUSSION

The response of the prostate to androgens appears to depend on how many cells are present in the prostate relative to the number normally present. If the normal cellular complement is present, as in normal animals or short term castrates (Figures 5 and 6), then even large doses of androgens cannot induce proliferation. This lack of effect is not due to failure of hormone to reach the prostate, since wet weights are greatly increased as a result of hormone administration, presumably due to stimulation of prostatic secretory function. On the other hand, after the number of cells in the prostate has fallen below the normal level, as in rats castrated longer than three days (Figures 7 and 8), the primary response of the prostate to restoration of androgen appears to be proliferation to restore the normal complement of cells. Once this has occurred, however, some homeostatic mechanism seems to switch off proliferation. It is difficult to say whether secretory activity of the prostate starts during the proliferative phase, or whether proliferation must be completed before secretion commences. This could be tested directly by assaying some parameter of prostatic function, such as production of citrate. In any case, it is clear that once the number of cells has returned to normal continued administration of androgens is an intense stimulus for secretory activity of the prostate. This sequence of events

- proliferation followed by expression of differentiated function - has been observed in a number of systems, as discussed in Chapter I. If it were possible to specifically block proliferation, this system would be an excellent one in which to test whether proliferation is a requirement for subsequent differentiation. Unfortunately, however, agents that block DNA synthesis or mitosis, such as hydroxyurea or colchicine, are probably too toxic for prolonged administration to rats in doses sufficient to completely block proliferation.

During androgen-stimulated regeneration of the 7 day castrate prostate (Figure 8), three phases can be distinguished. Firstly, there is a latent period of 24-36 hours during which no proliferative response can be detected, but, as discussed in the Introduction, the prostate is very active biochemically. During the latent period, preparations for DNA synthesis and cell proliferation must be taking place, and this is probably the key period to examine if one wishes to study the events regulating the onset of DNA synthesis. Secondly, there is a period of proliferation starting at 36-48 hours and continuing for about 72 hours. The kinetics of cellular proliferation during this time will be examined in Chapter V. Thirdly, there is a period of differentiation starting about 5 days after initiation of hormone treatment and continuing for at least 9 days, and presumably for as long

as hormone continues to be present. The events that occur around day 5 that shut off proliferation are undoubtedly of major importance insofar as understanding of the regulation of growth is concerned, and it may well be that the particular mechanisms operating at this time are the fundamental ones lacking in neoplastic cells.

The *in vitro* assay for DNA synthesis used routinely in this chapter can potentially serve as an excellent biochemical assay for the proliferative effects of androgens that would be simpler and more objective than the cytological assays used previously. The relative potencies of various hormones in stimulating DNA synthesis (Table 1) agree quite well with their effects on growth *in vivo* (Bruchovsky, 1971) and on prostatic explants in organ culture (Bobel et al, 1971). In addition, this assay can serve as a measure of antiandrogenic activity (Table 2). While the *in vitro* and *in vivo* assays for incorporation of thymidine show parallel changes in rate of DNA synthesis during hormone treatment (Figures 8 and 9), the two assays are of course not strictly comparable. At the peak rate of incorporation, at day 3 following initiation of hormone treatment, 13% of nuclei are labeled *in vivo* but only 3% *in vitro*. These differences are probably due to loss of cell viability prior to and during the *in vitro* incubation. Thus, although the *in vitro* assay cannot be used for measurement of absolute rates of DNA synthesis or of the true number of

cells involved, it can be used to compare rates of DNA synthesis as a result of various treatments.

Both the relative effects of various doses of testosterone and dihydrotestosterone on stimulation of the early DNA synthetic response (Figure 12) and the relative rates at which equal doses induce prostatic regeneration (Figures 8 and 11) suggest that dihydrotestosterone is a more potent androgen than testosterone. This finding is in agreement with the work of Schmidt et al (1972) and of Tuohimaa et al (1973). Coffey et al (1968) have found that testosterone propionate stimulates prostatic regeneration at the same rate as observed here with dihydrotestosterone, but at double the dose. Since Figure 12 demonstrates that doubling the dose of testosterone from 400 to 800 ug/100 g body weight has no effect on rate of DNA synthesis, it appears possible that testosterone propionate does not have the same androgenic properties as testosterone. This difference may well be due to the different solubilities of the two compounds and to the different vehicles used (sesame oil in the case of Coffey et al). In any case, if one assumes that dihydrotestosterone and testosterone share a common mechanism and site of action, then the findings presented in this chapter are consistent with the possibility that conversion of testosterone to dihydrotestosterone is a necessary step for expression of its proliferative effects.

V. KINETICS OF CELL PROLIFERATION DURING REGENERATION

INTRODUCTION

During androgen-stimulated growth of the rat prostate (Chapter IV), proliferation occurs at a maximal rate between days 2 and 5 after initiation of treatment of 7 day castrate rats with 400 ug dihydrotestosterone/100 g body weight. During this 72 hour period, the cell population undergoes 1.8 doublings with a doubling time of 40 hours, and then proliferation essentially ceases. In attempting to elucidate the mechanisms involved in this shutoff of proliferation, it would be useful to know the duration of the various cell cycle phases in the proliferating population, the fraction of cells involved in the proliferative response to hormone, and the average number of divisions a cell undergoes during the regeneration.

Previous studies of cell proliferation kinetics in androgen-stimulated tissues have concentrated on the various sex accessory glands of the mouse (Tuohimaa and Niemi, 1968; Morley and Wright, 1972; Morley et al, 1973). All of these studies have used the technique of fractional labeled mitoses to follow a cohort of labeled cells through successive mitoses. A pulse of ^{3}H-thymidine is administered to a group of animals. At various times, animals are killed,

tissues prepared for autoradiography, and the proportion of mitoses that are labeled is plotted against time. The time for the first labeled mitotic figures to appear corresponds to the duration of G2 (plus part of mitosis). The percentage of labeled mitoses increases to 100% for a period equal to the duration of S, and then falls to zero as the labeled cells move into G1. When the labeled cells re-enter the next mitosis, the percentage of labeled mitoses increases again. In practice, however, because the labeled cells do not progress synchronously through the cell cycle, a second wave of labeled mitoses is often not observed. In these cases the cell cycle time cannot be measured directly but must be calculated indirectly.

Tuohimaa and Niemi (1968) found that administration of testosterone propionate to two week castrate mice increases the labeling index of ventral prostate from 1.9% in untreated controls to 37%, and shortens the cell cycle time (Tc) from about 900 hours to 20 hours. The majority of the effect on Tc is due to shortening of G1 from 900 to 10 hours. In this study, however, a second wave of labeled mitoses was not observed, and values for G1 and Tc were calculated assuming steady state growth kinetics and a growth fraction of 1.0, assumptions that undoubtedly are invalid for an expanding population. Similar effects have been reported for seminal vesicle (Morley and Wright, 1972; Morley et al, 1973). Again, however, a second wave of

labeled mitoses was not observed and calculations were made assuming exponential growth, an assumption which also is probably incorrect. In addition, these workers have determined that the growth fraction (the proportion of cells proliferating at a given time) in mouse seminal vesicle varies with time after initiation of hormone treatment, rising from 0.14 at 24 hours to a peak of 0.64 at 48 hours, and then falling to 0.32 by 72 hours. By measuring grain count distributions following administration of ^{3}H-thymidine at 72 hours, they found that labeled cells undergo one division, that is, the average number of grains per cell is halved, but not a second division. Since regeneration is presumably almost complete by 72 hours, this finding is not very meaningful, and the experiment was not performed at earlier times in regeneration. In summary, studies using the technique of fractional labeled mitoses indicate that androgens increase the proportion of cells in the proliferative cell cycle and decrease the duration of this cycle, but the actual magnitude and relative importance of these effects is uncertain because of the many assumptions involved in the various calculations. In order to obtain answers to these questions, a more direct technique for studying the cell cycle is necessary.

Miller and Phillips (1969) have developed a technique for fractionating populations of particles on the basis of size by letting them settle through a shallow gradient under

the influence of gravity. The density of the gradient is low compared to that of the particles, so that their rate of movement depends directly on their size and shape. Because cells that have just divided (G1 cells) should be about half the size of those just preparing to divide (G2 cells), and cells in S phase should be of intermediate size, this technique offers the possibility of fractionating a proliferating population according to position of the cells within the cell cycle. McBride and Peterson (1970) have reported that such fractionation of exponentially growing Hela cells is indeed possible.

The work reported in this chapter concerns the study of cell proliferation kinetics in regenerating rat prostate using the technique of velocity sedimentation under unit gravity to fractionate the population according to position in the cell cycle. Isolated nuclei rather than whole cells are used in this study because of the likelihood that variations in nuclear size through the cell cycle would be more pronounced than variations in the size of the whole cell, and because of the difficulty in isolating intact cells from a solid tissue such as the prostate.

MATERIALS AND METHODS

Treatment of animals. Seven day castrates were treated with daily doses of 800 ug dihydrotestosterone/100 g body weight as described in Chapter II. This is double the dose

used in most experiments in Chapter IV, and was used because it produces a more reproducible growth response.

[Methyl-^{3}H] thymidine was injected intraperitoneally in saline as described in Chapter II at a dose level of 5 uCi/100 g body weight. At this dose, the isotope has no deleterious effects on prostatic growth, since prostatic weights and nuclear yields are the same as for control animals that have not been injected with labeled thymidine. In preliminary experiments it was found that, at dose levels greater than 10 uCi/100 g body weight, these parameters were consistently less than control values at times longer than 24 hours after injection.

Preparation of nuclei. "Triton-purified" nuclei were prepared as described in Chapter II.

Fractionation of nuclei. The apparatus for velocity sedimentation was purchased from O.H. Johns Scientific (Toronto, Ont.) and modified by addition of a 4° water jacket around the sedimentation chamber (Figure 13). The sedimentation chamber had a diameter of 16.9 cm and hence a volume of 22.4 ml per vertical mm. All bovine serum albumin (BSA) solutions were prepared in PBS containing 0.02% (v/v) Triton X-100 (Sigma) to prevent aggregation of nuclei, and were filtered through a 0.22um Millipore filter (Millipore Corp., Montreal, P.Q.). The 1% (w/v) and 2% (w/v) BSA chambers each contained 600 ml of solution, and the 0.5%

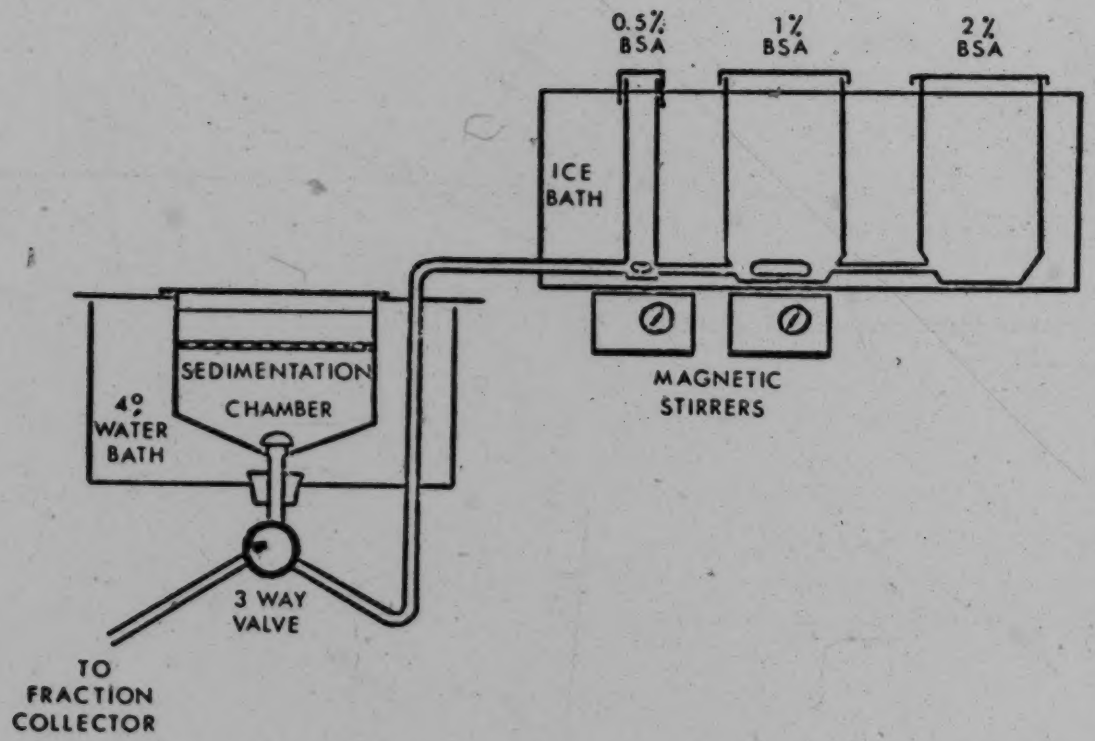

Figure 13. Apparatus for fractionation of nuclei by velocity sedimentation under unit gravity.

(w/v) BSA chamber contained liquid to the same level as the other two.

The nuclear suspension was diluted to a concentration of 3.0×10^6/ml in PBS containing 0.3% (w/v) BSA, and unless stated otherwise the volume applied to the sedimentation chamber was 20 ml. The gradient was loaded at a rate of about 30 ml/min, allowed to stand for 8 hours (starting from the time liquid reached the top of the cone on loading), and then unloaded at 15-20 ml/min with fractions of 10.5-11.5 ml collected using a Brinkmann SFZ fraction collector (Brinkmann Instruments, Toronto, Ont.).

Sedimentation velocity was computed using the equation

$$SV = [(Nt-Nf)D]/[St+Ct(Nf/Nt)]$$

where SV = sedimentation velocity for fraction Nf
Nf = fraction number
Nt = total number of fractions
D = depth/fraction = fraction volume/(22.4 ml/mm)
St = standing time (from top of cone on loading to start of unloading)
Ct = collection time (from start of unloading to top of cone)

Distribution of nuclei. An aliquot of each fraction was diluted with filtered PBS and 50 ul counted using a Coulter counter model B (Coulter Electronics, Hialeah, Fla.) fitted with a 50 um aperture. Optimal settings were determined by plotting the size distribution of unfractionated nuclei at various settings, and were 0.707 for 1/APC, 2 for 1/AMP, 70 for gain, 32H for matching switch, 20 for lower threshold,

and 100 for upper threshold. Recovery of nuclei from the gradient was about 70%.

Distribution of radioactivity. After removal of an aliquot for counting of nuclei, the rest of each fraction was deposited onto a 0.45um Millipore filter (Millipore Corp.). The filters were washed with 5 ml cold PBS and then twice with 5 ml cold 5% (w/v) trichloroacetic acid, and dried overnight at 37°. Unless DNA was to be measured (see below) the filters were counted in a scintillant containing 4 g 2,5-diphenyloxazole (Amersham-Searle Corp., Arlington Heights, Ill.) per litre scintillation grade toluene (Fisher). Backgrounds were taken as the mean + 2 standard deviations of five blank filters, and were 30-35 for tritium. Recovery of radioactivity from the gradient was 80-90%.

Double isotope counting was performed using two channels of the Beckman LS250 scintillation counter. A variable isoset was used for the ^{3}H channel, with lower window set at 0 and upper window at 3. The preset ^{14}C isoset was used for the ^{14}C channel. Background for ^{3}H was 5 cpm and efficiency about 13%; background for ^{14}C was 9 cpm and efficiency about 60%. Using ^{3}H and ^{14}C standards, spillover of ^{14}C into the ^{3}H channel was determined to be 2.6%, and ^{3}H counts were corrected for spillover by subtracting 2.6% of ^{14}C counts for that sample. Spillover of ^{3}H counts into the ^{14}C channel was negligible.

Distribution of DNA. If DNA was to be measured, 2.0 ml 1.6 N perchloric acid were added to the dried filters and DNA extracted by heating at 70° for 20 minutes. The filters were removed; 1.5 ml of the perchloric acid extract was assayed for DNA using the diphenylamine procedure as described in Chapter II, and the rest of the extract (about 0.4 ml) was counted as described in Chapter II for aqueous samples.

RESULTS

Fractionation of nuclei according to phase of the cell cycle. When a population of nuclei from 7 day castrate prostates that have been regenerating for 72 hours is allowed to settle through a shallow BSA gradient under unit gravity, two peaks of material are observed (Figure 14). Microscopic examination of the slowly sedimenting peak (about 0.4 mm/hour) shows it to consist of debris. It contains no DNA and no radioactive thymidine and for purposes of clarity will be omitted from subsequent figures. Nuclei are distributed as a main peak centered at 2.0 mm/hour and skewed towards larger sedimentation velocities. After a 1 hour pulse *in vivo* with [methyl-^{3}H] thymidine just prior to killing, label is found in a peak heavier than that of the majority of nuclei, with a maximum at about 2.7-3.0 mm/hour. Nuclei sedimenting between 1.3 and 2.2-2.3 mm/hour contain the diploid amount of DNA and little labeled

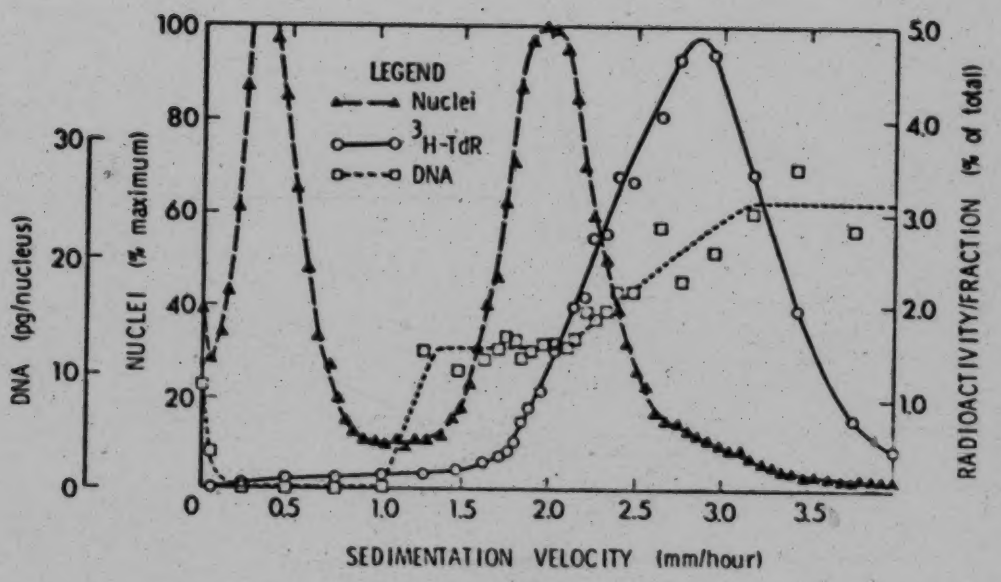

Figure 14. Fractionation of nuclei according to phase of the cell cycle. 7 day castrates were treated for 72 hours with daily doses of 800 ug dihydrotestosterone/100 g body weight. One hour prior to killing, animals were injected with 5 uCi [methyl-^{3}H] thymidine/100 g body weight. Nuclei were isolated, and a total of 1.2×10^8 in a volume of 40 ml applied to the gradient. The distribution of particles after 8 hours is shown for each fraction as a percentage of the value of the peak at 2 mm/hour. Distribution of radioactivity is expressed for each fraction as a percentage of total radioactivity recovered, and DNA is shown as pg/nucleus. Fractions which contained a small number of nuclei were pooled to obtain sufficient DNA.

thymidine, and accordingly are considered to be in the G0 and G1 phases of the cell cycle. Nuclei sedimenting between 2.2-2.3 mm/hour and about 3.1 mm/hour contain increasing amounts of DNA and most of the labeled thymidine, characteristic of nuclei in the process of DNA replication, that is, the S phase of the cell cycle. Finally, nuclei sedimenting more rapidly than 3.1 mm/hour contain twice the diploid amount of DNA and little labeled thymidine, and are considered to be in G2.

Progression of nuclei through the cell cycle. Seven day castrates were pulse labeled with an intraperitoneal injection of [methyl-^{3}H] thymidine at 48 hours after initiation of hormone treatment (hereafter referred to as time zero), and the progression of the labeled cells through the cell cycle followed for the subsequent 48 hours. Hepatic metabolism of the injected isotope produces a chase effect, with label apparently available to the prostate for a period of time of somewhat less than one hour.

Figure 15 shows the results during the first 12 hours. After 1 hour (Figure 15B), labeled nuclei are in the S phase region of the gradient, and after 2 hours label has shifted to heavier regions. By 4 hours (Figure 15C), some label has appeared in the G1 region, representing nuclei that have completed their first division, while the main peak occurs in S+G2 nuclei. After 8 hours (Figure 15D), the majority of the label is in the G1 area, and by 12 hours the shift is

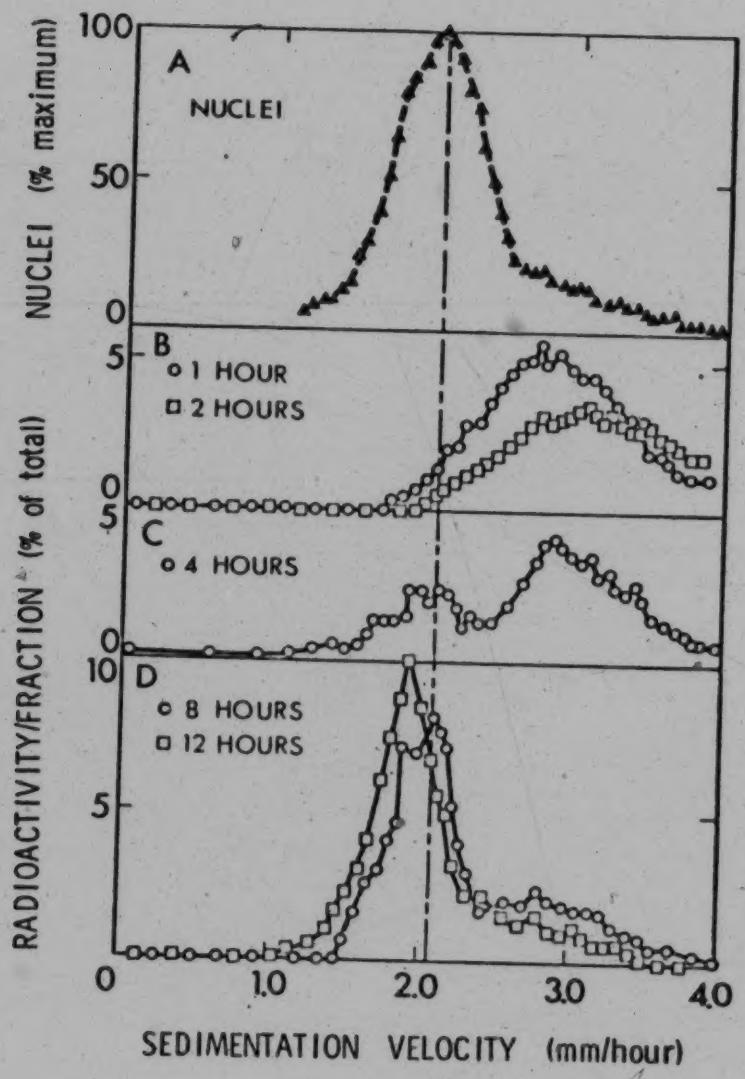

Figure 15. Distribution of label between 1 and 12 hours. 7 day castrates were injected at 24 hour intervals with 800 ug dihydrotestosterone/100 g body weight. Along with the third injection at 48 hours, the animals were injected intraperitoneally with 5 uCi [methyl-^{3}H] thymidine/100 g body weight. At various times after injection of isotope rats were killed and prostatic nuclei fractionated as described in Methods. The distribution of nuclei is expressed for each fraction as a percentage of the peak value, and of radioactivity as the percentage of the total recovered in each fraction.

almost complete and little label remains in the S+G2 regions. At 12 hours the peak of radioactivity is actually slower than the peak of nuclei; this may indicate that nuclei which have just divided are smaller than the majority of G1 nuclei.

Results for times later than 12 hours (Figures 16 and 17) are plotted as relative specific activities, i.e., the specific activity of a fraction (cpm/10^6 nuclei) relative to the specific activity of unfractionated nuclei, in order to demonstrate enrichment or depletion of label in various phases of the cell cycle. Figure 16B shows the data for 12 hours replotted in this manner. Enrichment of label occurs in "early" G1 and depletion in "late" G1. The situation is similar at 16 hours (Figure 16C). At this time the amount of label in the S+G2 regions of the gradient reaches a minimum of 12.4% of the total, a value which may represent the level of contamination of this region by G1 nuclei. At 20 hours (Figure 16D) the S+G2 region is somewhat enriched in label, and by 24 hours (Figure 16E) there has evidently been a shift of label back into this region. At this time, 31.5% of the label is present in the S+G2 region of the gradient, an increase of 19% over the amount present at 16 hours. After 24 hours (Figure 17) the S+G2 region continues to be enriched in label, especially at 42 hours (Figure 17D), but by 48 hours (Figure 17E) there is very little label remaining in this region.

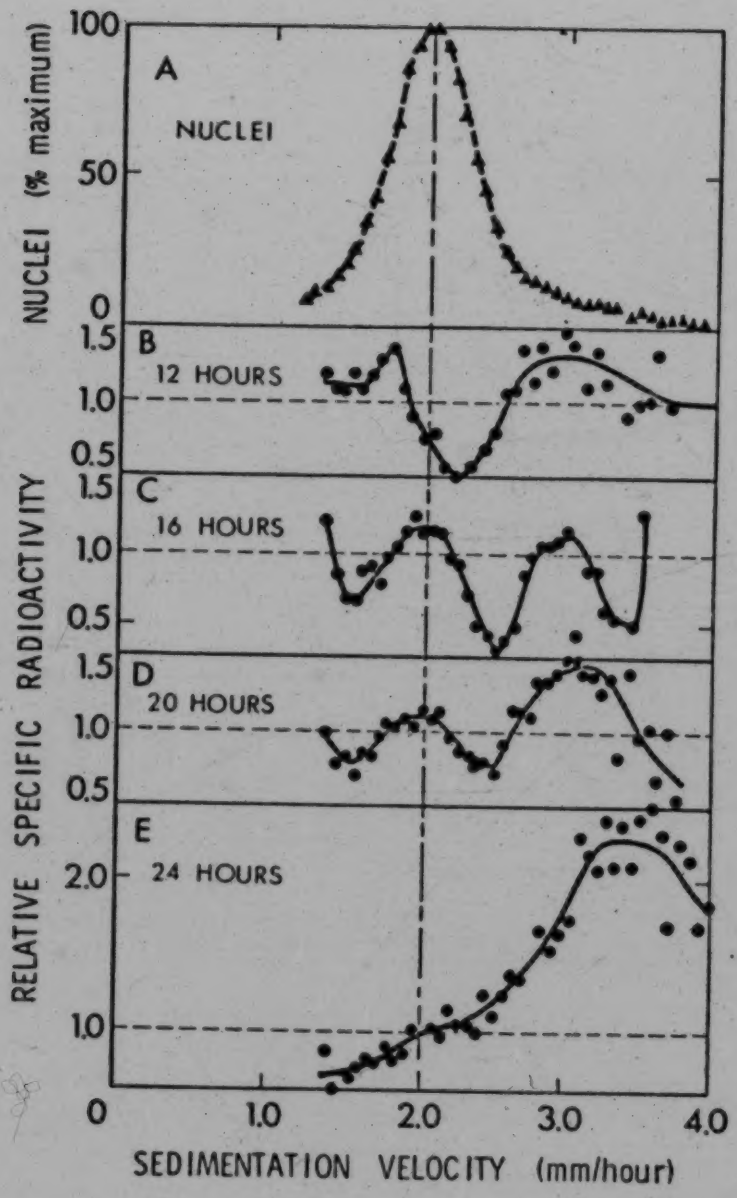

Figure 16. Distribution of label between 12 and 24 hours. 7 day castrates were injected with daily doses of 800 ug dihydrotestosterone/100 g body weight. Along with the third injection at 48 hours the animals were injected intraperitoneally with 5 uCi [methyl-^{3}H] thymidine/100 g body weight. At various times after injection of isotope rats were killed and prostatic nuclei fractionated as described in Methods. The distribution of nuclei is expressed for each fraction as a percentage of the peak value, and radioactivity as specific activity relative to that of unfractionated nuclei set at 1.0.

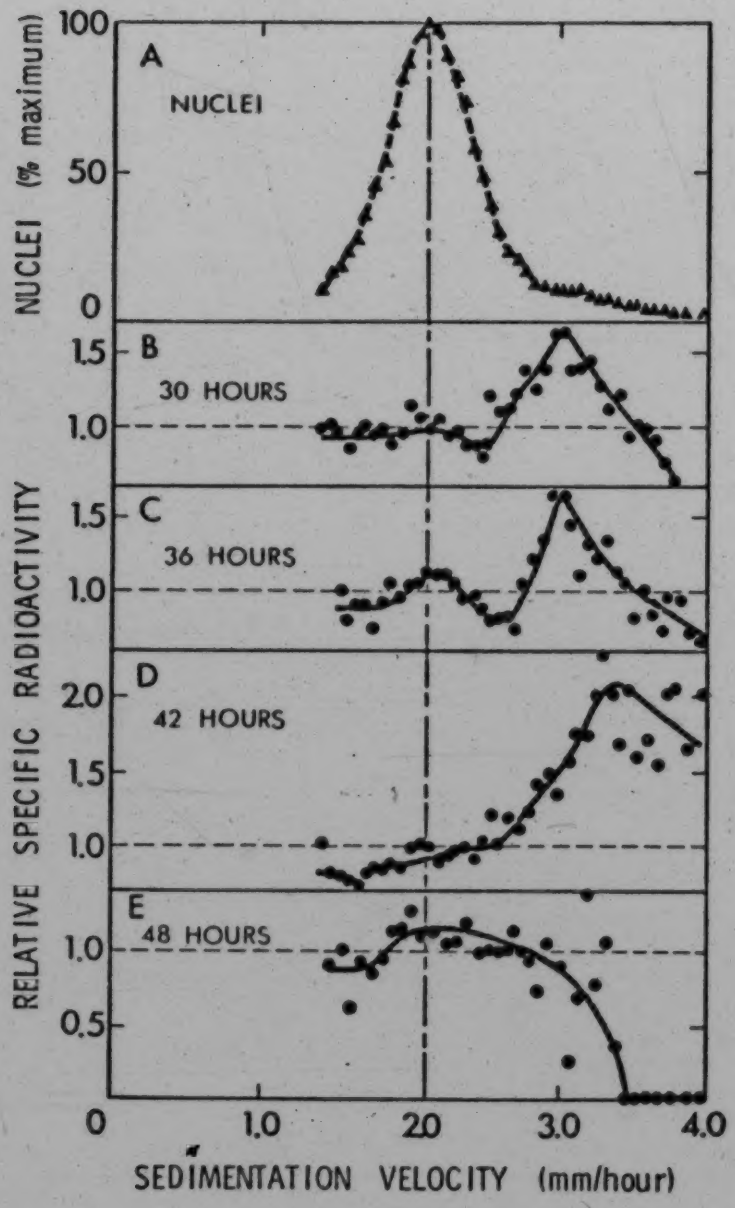

Figure 17. Distribution of label between 30 and 48 hours. 7 day castrates were injected with daily doses of 800 ug dihydrotestosterone/100 g body weight. Along with the third injection at 48 hours the animals were injected intraperitoneally with 5 uCi [methyl-^{3}H] thymidine/100 g body weight. At various times after injection of isotope rats were killed and prostatic nuclei fractionated as described in Methods. The distribution of nuclei is expressed for each fraction as a percentage of the peak value, and radioactivity as specific activity relative to that of unfractionated nuclei set at 1.0.

Persistence of DNA synthesis after 16 hours. During the period after 16 hours, by which time the majority of cells have completed their first division, the bulk of the label at any one time remains in the G1 area of the gradient (Figures 16 and 17), indicating that synchronous re-entry of cells into S phase does not occur. In order to ascertain that failure of the majority of label to shift back to the S phase region is not due to a change in properties of S phase nuclei or to a cessation of DNA synthesis after one round of division, regenerating prostates which had been pulse labeled with [methyl-^{3}H] thymidine at zero time (i.e., 48 hours after initiation of treatment) were pulse labeled with [methyl-^{14}C] thymidine at 24 hours. As shown in Figure 18, while the bulk of the ^{3}H label is found in the G1 region of the gradient, as expected from previous results, the ^{14}C label occurs in the S phase area. Hence there is considerable DNA synthesis occurring during the period after 16 hours, and S phase nuclei at this time have the same sedimentation properties as those at earlier times.

DISCUSSION

The technique of cell cycle analysis by velocity sedimentation is based on the increasing size of nuclei as cells progress through the cell cycle. From Figure 14 it is evident that G2 nuclei, which sediment faster than 3.1 mm/hour, are essentially twice as large as G1 nuclei, which

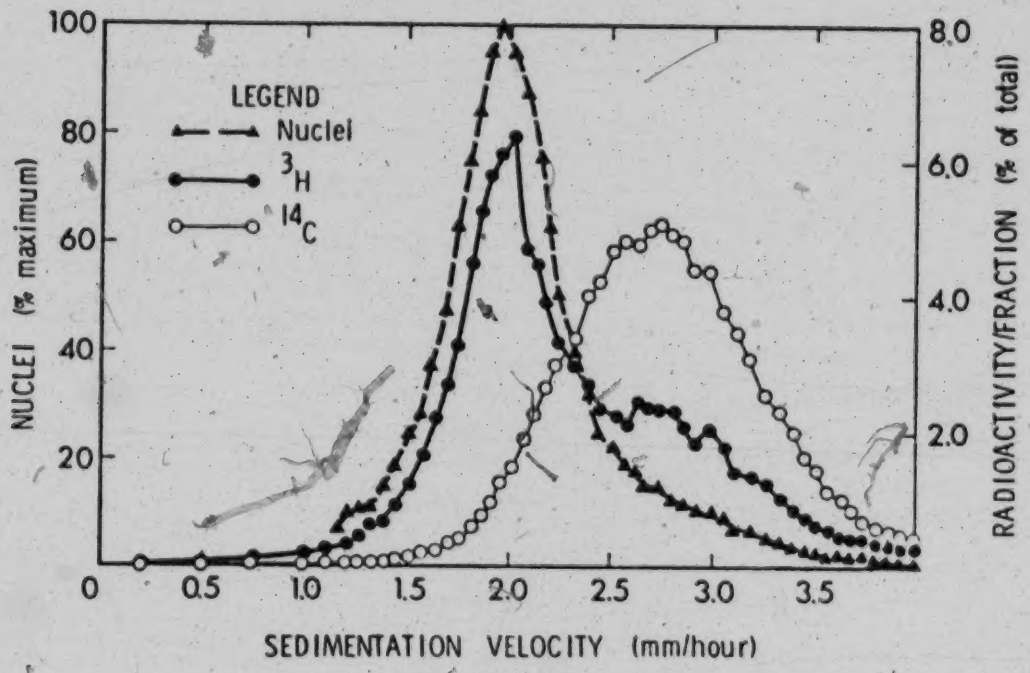

Figure 18. Persistence of DNA synthesis at 24 hours. 7 day castrates were treated at 24 hour intervals with 800 ug dihydrotestosterone/100 g body weight. Along with the third injection at 48 hours the rats were injected intraperitoneally with 5 uCi [methyl-^{3}H] thymidine/100 g body weight. 23 hours later the rats were injected intraperitoneally with 5 uCi [methyl-^{14}C] thymidine/100 g body weight. One hour after injection of ^{14}C (24 hours after ^{3}H injection) the animals were killed and prostatic nuclei fractionated as described in Methods. Distribution of nuclei is shown for each fraction as the percentage of the peak value. Double isotope counting was done as in Methods and results are shown as the percentage of the total recovered in each fraction.

have a sedimentation velocity of about 2 mm/hour, and that S phase nuclei have intermediate sedimentation velocities and thus intermediate sizes. Hence it is possible to follow all phases of the cell cycle simultaneously, and nuclei from various stages can be recovered for biochemical analysis. The one major drawback - the long periods of time required for good separation of slowly moving particles such as nuclei - could be overcome by the use of a low speed centrifugation system instead of unit gravity, and this would probably result also in improved resolution of the cell cycle phases.

By measuring the appearance of label in the G1 region of the gradient at various times after labeling of S phase nuclei (Figure 15), it is possible to estimate the duration of the various cell cycle phases. The time required for the first appearance of label in this region will correspond to the minimum duration of G2+mitosis. Since about 25% of label is present in G1 by 4 hours (Figure 15C), a reasonable estimate for G2+mitosis would be 2-3 hours. It will take a length of time equal to the duration of S+G2+mitosis for all the label to be transferred to the G1 region. After 8 hours (Figure 15D), 80% of the label is in the G1 region, and by 12 hours this value has reached 85%. Thus the length of S+G2+mitosis is somewhat greater than 8 hours, leading to an estimate of about 7 hours for the S phase. The first reappearance of label in the S phase region (Figure 16)

indicates the minimum duration of the cell cycle, in this case about 20 hours. From the values of 2-3 hours for G2+mitosis, 7 hours for S, and 20 hours for the cell cycle time (Tc), G1 can be determined to be approximately 10-11 hours. The accuracy of these estimates could be improved if desired by analysis at more frequent intervals after the pulse of labeled thymidine.

At 16 hours after a pulse of ^{3}H-thymidine (Figure 16C), 12.4% of the label is found in the S+G2 regions of the gradient. Between 16 and 48 hours the amount of label in this region is greater than 12.4%. If one takes the difference between these values to represent label that is re-entering a second S phase and assumes that the 6 hour interval between experiments is sufficiently long compared to the duration of the S phase (7 hours) such that each cell is counted only once in its passage through S, then the sum of the above values will correspond to the proportion of the labeled population that undergoes a second round of division during this interval. This value is 50.9%, which may indicate that one daughter cell resulting from each division differentiates while the other continues to divide. In any case, it certainly appears that only a fraction of daughter cells re-enters the proliferative cell cycle within one cell cycle time after division. Thus growth kinetics in the regenerating prostate are not exponential. The finding that 50% of labeled nuclei undergo a second round of division

would be consistent with steady state kinetics; however, as will be discussed below, maintenance of the observed rate of growth requires recruitment of previously nonproliferating cells, and Figure 18 demonstrates that this recruitment occurs. Hence cell proliferation kinetics in regenerating rat prostate are neither strictly exponential nor strictly steady state.

A cell cycle time of 20 hours together with a population doubling time of 40 hours leads to an estimate of 0.41 for the growth fraction (GF). At 48 hours after initiation of hormone treatment, examination of autoradiographs of prostatic slices following continuous labeling with ^{3}H-thymidine reveals the labeling index to be 16% and to be increasing at the rate of about 1% per hour, and the mitotic index to be 2.1% (it is interesting to note that, if one calculates the percentage of nuclei found in the S+G2 region of the gradient, it averages out to 16.3%, in excellent agreement with the labeling index determination). The fact that the labeling index is initially 16% and is increasing at 1% per hour indicates that after one cell cycle time (20 hours) the growth fraction will be 0.36. If one assumes steady state kinetics, that is, a random distribution of nuclei around the cell cycle, then the following equations can be used to calculate the growth fraction (Epifanova, 1966):

$$GF = LI \times Tc/Ts \quad \text{or} \quad GF = MI \times Tc/Tm$$

where LI = labeling index = 16%
Tc = cell cycle time = 20 hours
Ts = duration of S = 7 hours
MI = mitotic index = 2.1%
Tm = duration of mitosis = 1 hour

The growth fraction is 0.46 using labeling index or 0.42 using mitotic index data. Alternatively, assuming exponential growth, the growth fraction can be calculated from the following equation (Cleaver, 1967):

$$GF = LI/[(\exp(Ts\ln 2/Tc)-1)\exp(Tq\ln 2/Tc)]$$

where LI = labeling index = 16%
Ts = duration of S = 7 hours
Tc = cell cycle time = 20 hours
Tq = duration of G2+0.5mitosis = 2 hours

In this case, the value for GF is 0.54. This value does not agree very well with the other four values; this may indicate that the proliferation kinetics are closer to steady state than to exponential. In summary, therefore, several different methods of calculation lead to an estimate of 0.4 for the fraction of cells involved in the initial proliferative response to dihydrotestosterone.

The growth fraction is undoubtedly changing with time during the course of regeneration, since by 72 hours after initiation of treatment the labeling index reaches a maximum of about 20%, as determined by examination of autoradiographs of prostatic slices. Assuming the cell cycle parameters do not change, this would correspond to a growth fraction of just less than 0.6. However, after this time the labeling index decreases rapidly, so that 0.4 is probably a

reasonable estimate for the average growth fraction during prostatic regeneration, and a growth fraction of this magnitude is capable of maintaining cell production at the rates observed. With a 20 hour cell cycle time, almost four cycles of division would be required to repopulate the prostate. Assuming that the initial growth fraction is 0.4 and that 50% of daughter cells do not re-enter the proliferative cell cycle after each round of division, it is evident that essentially all the cells present in the 7 day castrate prostate must proliferate at some time during prostatic regeneration.

The results presented herein are in good agreement with some of the findings of previous studies using the technique of fractional labeled mitoses (Tuohimaa and Niemi, 1968; Morley and Wright, 1972; Morley et al, 1973). The values for cell cycle parameters are almost identical to those reported by Tuohimaa and Niemi (1968) despite differences in species studied (mouse vs rat) and hormone administered (testosterone propionate vs dihydrotestosterone). The work of Morley and Wright (1972) and of Morley et al (1973) indicates that the proliferative response of mouse seminal vesicle to testosterone propionate is similar to that of the rat prostate to dihydrotestosterone, except that it is more rapid. Allowing for the differences in time course, the labeling index, mitotic index, duration of cell cycle phases, and growth fraction at various times during

regeneration all agree quite well with the values determined herein for rat prostate. A major point of disagreement is the finding by Morley and Wright (1972) that cells labeled by a pulse of ^{3}H-thymidine undergo one division but not a second, in contrast to the result reported above that about 50% of label re-enters the proliferative cell cycle. A likely explanation for this disagreement is that Morley and Wright (1972) labeled their animals at a time when regeneration is almost complete, while in the present study isotope was administered at the beginning of regeneration. A second possibility is that of radiation damage. Because of the relative inefficiency of autoradiography, rather high doses of ^{3}H-thymidine must be administered in order to accumulate a sufficient number of silver grains for determination of labeled mitoses in a reasonable period of time. Bond and Feinendegen (1966) have reported that it may take as few as 20 intranuclear tritium disintegrations per cell to produce minimally detectable effects on rat bone marrow. In the paper of Morley and Wright (1972), it can be calculated from data presented that, even assuming an autoradiographic efficiency of 10% (probably a maximal value), this number of disintegrations will occur in about 16 hours, less than one cell cycle time. Thus the cells may undergo one division normally but subsequent proliferation may well be impaired by radiation damage. In the current study, because the detection method is liquid scintillation counting of large numbers of nuclei, such low levels of

radioactivity could be employed that it would take over 300 hours for an average labeled nucleus to accumulate 20 disintegrations. Hence cell-cycle analysis by sedimentation velocity as opposed to fractional labeled mitoses greatly extends the period during which one can follow labeled cells without the danger of radiation damage.

To summarize the findings in this chapter, the following conclusions can be drawn with regard to cellular proliferation kinetics in regenerating rat prostate: firstly, all cells in the 7 day castrate prostate seem to be involved in regeneration of the organ, and, secondly, any given cell can apparently divide from one to four times.

VI. ROLE OF CYCLIC AMP IN ANDROGEN-STIMULATED PROLIFERATION

INTRODUCTION

Adenosine 3':5' cyclic-monophosphate (cyclic AMP) is an intracellular mediator that, since its discovery by Sutherland, has been shown to act as a "second messenger" by which certain regulatory substances, e.g., catecholamines and certain peptide hormones, exert their effects on intracellular metabolism (Robison et al, 1971). Cyclic AMP is formed from ATP by the membrane bound enzyme adenylate cyclase and regulates metabolism by influencing the activity of various enzymes, e.g., glycogen phosphorylase (Krebs et al, 1966), glycogen synthetase (Larner et al, 1968) and various protein kinases (e.g., Walsh et al, 1968).

Recently, much attention has focussed on the possible role of cyclic AMP in the regulation of cell proliferation. Evidence for such a role is that levels of cyclic AMP appear to be inversely correlated with rates of proliferation, both in normal cells (Macmanus et al, 1972; Burger et al, 1972; Millis et al, 1972; Kram et al, 1973) and in transformed cells (Otten et al, 1972; Sheppard, 1972), and that proliferation of both normal cells (Willingham et al, 1972; Bombik and Burger, 1973; Froehlich and Rachmeler, 1974) and transformed cells (Smets, 1972; Paul, 1972; van Wijk et al,

1973; Thomas et al, 1973; Teel and Hall, 1973) is inhibited by increased intracellular levels of cyclic AMP. Thus cyclic AMP has been considered to be an inhibitor of cell proliferation. However a report has appeared indicating that in chick embryo fibroblasts cyclic AMP stimulates growth (Hovi and Vaheri, 1973).

Insofar as steroid hormones are concerned, their ability to enter cells and be taken up into the nucleus obviates the necessity for a second messenger such as cyclic AMP. Nevertheless a few reports have appeared indicating that cyclic AMP can mimic the effects of androgens in stimulating certain carbohydrate metabolizing enzymes in the prostate (Singhal et al, 1971; Mangan et al, 1973), leading to the hypothesis that androgenic effects may be mediated by cyclic AMP (Singhal et al, 1971). However the effects of cyclic AMP on prostate are insignificant relative to the effects of androgens (Mangan et al, 1973), and androgens do not appear to stimulate adenylate cyclase, as would be expected if this hypothesis were true (Rosenfeld and O'Malley, 1970; Liao et al, 1971; Mangan et al, 1973).

The proliferative response of the castrate rat prostate to androgens (Chapter IV) is an excellent model system for the study of agents that affect steroid-stimulated cell proliferation. Accordingly, studies have been performed to determine whether cyclic AMP can mimic or modify the proliferative effects of dihydrotestosterone.

MATERIALS AND METHODS

Treatment of animals. Adenosine 3':5' cyclic-monophosphate (Sigma) or N^6,$O^{2'}$-dibutyryl cyclic AMP (Sigma) and theophylline (Sigma) were administered intraperitoneally in 1.0 ml sterile saline containing in solution 10 mg cyclic AMP or dibutyryl cyclic AMP plus in suspension 10 mg sonicated theophylline. This was the protocol used by Singhal et al (1971). It has been assumed that dibutyryl cyclic AMP can penetrate prostatic cells when administered by this route and that it has the same biological properties as cyclic AMP. However, these assumptions have not been tested. Dihydrotestosterone in a dose of 400 or 800 ug/100 g body weight was injected as described in Chapter II.

RESULTS

Effect of cyclic AMP or dibutyryl cyclic AMP on prostatic growth. In order to test whether cyclic AMP can induce prostatic growth, 7 day castrate rats were treated with two daily doses of cyclic AMP or dibutyryl cyclic AMP, and prostatic weight, number of nuclei and rate of DNA synthesis *in vitro* measured. Results are shown in Table 3. It is evident that neither cyclic AMP nor dibutyryl cyclic AMP has any significant effects on any of the parameters tested, while dihydrotestosterone has very marked effects even after this short period of treatment.

TREATMENT	PROSTATIC WEIGHT (mg/prostate)	NUCLEI/PROSTATE x10^{-6}	DNA SYNTHESIS (dpm/ug DNA)
Saline	49±5	4.2±0.4	4.2±0.7
Cyclic AMP	47±3	3.1±0.4	4.3±0.7
Db cyclic AMP	45	2.4	2.9
DHT	87±11	7.7±0.2	716.1±11.5

Table 3. Effect of cyclic AMP or dibutyryl cyclic AMP on prostatic growth. Seven day castrate rats were injected with cyclic AMP or dibutyryl cyclic AMP as in Methods. Controls were injected with saline. The injections were repeated 24 hours later. 48 hours after the first injection, prostates were removed and rate of DNA synthesis assayed *in vitro* as described in Chapter II. Results are shown as mean±S.E. where at least three experiments were done, or else as single determinations. The values for animals treated with dihydrotestosterone as in Chapter IV are shown for comparison. Abbreviations used are DHT for dihydrotestosterone and Db cyclic AMP for dibutyryl cyclic AMP.

Effect of dibutyryl cyclic AMP on dihydrotestosterone-stimulated prostatic growth. To determine whether dibutyryl cyclic AMP enhances or inhibits the effects of dihydrotestosterone on prostatic growth, 7 day castrates were treated with hormone alone or with hormone plus dibutyryl cyclic AMP for 2 or 5 days. As seen in Table 4, dibutyryl cyclic AMP has no significant effects on dihydrotestosterone-stimulated increase in weight or in the number of nuclei per prostate. However, when rate of incorporation of radioactive thymidine was assayed, a marked inhibition following dibutyryl cyclic AMP treatment was noted (Table 5). When dibutyryl cyclic AMP was administered to animals in vivo, a 27-30% inhibition in incorporation of [methyl-^{3}H] thymidine in vitro was observed after either 2 or 5 days of treatment, compared to tissue treated with dihydrotestosterone alone. When rate of incorporation in vitro was assayed in tissue treated with hormone alone but with dibutyryl cyclic AMP present in the incubation mixture, a very marked inhibition (82%) was found. This very rapid effect of dibutyryl cyclic AMP makes it unlikely that its effects are at the level of DNA synthesis itself. Finally, to ensure that the effects of dibutyryl cyclic AMP on incorporation of thymidine are not an artefact of the in vitro incubations, rate of incorporation of thymidine was assayed in vivo and an even greater inhibition (63%) was observed.

TREATMENT	DAYS	PROSTATIC WEIGHT (mg/prostate)	NUCLEI/PROSTATE x10^{-6}
DHT+saline	2	93±4	8.3±1.2
DHT+Db cyclic AMP	2	90±5	8.6±0.5
DHT+saline	5	160±25	18.8±2.3
DHT+Db cyclic AMP	5	186±37	19.6±1.2

Table 4. Effect of dibutyryl cyclic AMP on dihydrotestosterone-stimulated prostatic growth. Seven day castrate rats were injected simultaneously with daily doses of dihydrotestosterone+saline or dihydrotestosterone+dibutyryl cyclic AMP. Abbreviations used are DHT for dihydrotestosterone and Db cyclic AMP for dibutyryl cyclic AMP. At 2 days or 5 days after initiation of treatment (24 hours after the last injections), prostates were removed and weighed and nuclear content determined as described in Chapter II. Results are shown as mean±S.E. for at least three experiments.

TREATMENT	DAYS	INCORPORATION OF ^{3}H-THYMIDINE (dpm/ug DNA)	INHIBITION RELATIVE TO CONTROL (%)
		in vitro	
DHT+saline	2	513.6±87.2	0
DHT+Db cyclic AMP	2	376.3±61.5	27
DHT+saline + Db cyclic AMP in vitro	2	93.2	82
DHT+saline	5	463.8±38.2	0
DHT+Db cyclic AMP	5	328.1±8.2	30
		in vivo	
DHT+saline	2	194.2	0
DHT+Db cyclic AMP	2	72.2	63

Table 5. Inhibition of [methyl-^{3}H] thymidine incorporation by dibutyryl cyclic AMP. Seven day castrates were injected simultaneously with daily doses of dihydrotestosterone+saline or dihydrotestosterone+dibutyryl cyclic AMP. At 2 days or 5 days after initiation of treatment (24 hours after the last injections), incorporation of thymidine was assayed *in vitro* or *in vivo* as described in Chapter II. In one experiment, animals were treated with dihydrotestosterone alone, and dibutyryl cyclic AMP plus theophylline were added to the incubation mixture *in vitro* at a concentration of 1 mM each. Abbreviations used are DHT for dihydrotestosterone and Db cyclic AMP for dibutyryl cyclic AMP. Results are shown as mean±S.E. for at least three experiments or else as single determinations.

DISCUSSION

The findings presented in this chapter show that cyclic AMP is not involved in androgen-stimulated cell proliferation, either as a mediator or as a modulator. It neither stimulates prostatic growth itself nor modifies the growth response of prostate to dihydrotestosterone. These results are in agreement with the findings that androgens do not stimulate adenylate cyclase (Rosenfeld and O'Malley, 1970; Liao et al, 1971; Mangan et al, 1973), and that the level of cyclic AMP remains constant during atrophy and regeneration of the prostate (Craven et al, 1974). The inhibition by cyclic AMP of incorporation of thymidine is apparently not at the level of DNA synthesis itself, since production of DNA is not affected, but may be due to an effect on uptake or utilization of exogenous precursor. This point is important insofar as rate of cell proliferation is routinely measured by rate of incorporation of labeled thymidine.

Therefore, it appears that if cyclic AMP does have any restricted effects on the prostate, these effects are insignificant relative to the wide spectrum of biochemical changes elicited by androgens.

VII. CONCLUSIONS

The responses of the rat prostate to depletion and restoration of androgens have been developed as a model system for the study of steroid hormone action and the fundamental biological processes of tissue atrophy, cell proliferation, and cell differentiation. The advantages of this system include the ready availability of sufficient quantities of tissue for biochemical studies, the ability to induce the various responses with a known and reproducible time course by hormonal manipulation, the availability of hormone antagonists as a test for specificity of the responses, the wealth of biochemical and structural information accumulating concerning prostatic function, the apparent homogeneity of response to hormone in the prostatic cell population, and the fact that androgens appear to be the sole hormones on which normal prostatic function depends. In addition, understanding the mechanisms involved in regulation of normal prostatic growth would contribute to knowledge about regulation of growth in many other normal tissues, and would provide a standard against which to compare the abnormal growth conditions of prostatic carcinoma and benign prostatic hyperplasia.

UPTAKE AND METABOLISM OF ANDROGENS IN RELATION TO PROLIFERATIVE EFFECTS

It has been shown in Chapter IV that the response of the prostate to androgens depends on how long animals have been castrated prior to initiation of treatment. In animals castrated less than three days, the cellular complement has not fallen below normal, and the response to hormone is secretion rather than proliferation. In long term castrates (four days or more), the initial response is proliferation until the normal cellular complement has been restored, and then the response switches to secretion. Thus in studies of androgen action, the duration of castration must be chosen so as to produce the response one wishes to study.

The relative effects of the various androgens and their metabolites have never been clearly defined because most studies have used prostatic weight as an index of potency. The systems developed in Chapter IV provide the ability to test androgens independently for their potency with regard to stimulation of proliferation and of secretion. One point that has emerged from studies of the relative potency of various androgens is that these effects may be due to differences in the amount of hormone actually reaching the target tissue after injection of equal doses rather than to a true difference in potency. For example, testosterone propionate appears to be almost as active an androgen as dihydrotestosterone, while testosterone is less potent

(Coffey et al, 1968; Schmidt et al, 1972; Tuohimaa et al, 1973; plus results in Chapter IV). This may be due to the fact that the solubilities of testosterone propionate and of dihydrotestosterone are similar, while testosterone is less lipid soluble. Thus one must ensure that, when potencies are compared, the same mode of administration has been used, and one must bear in mind that in different injection vehicles the relative potencies of androgens may change.

The suggestion has been made (Robel et al, 1971) that the various androgen metabolites have different effects on the prostate. These findings and the results presented in Chapter IV indicate that dihydrotestosterone is more potent as a stimulus for proliferation than testosterone, but that testosterone may be a stronger stimulus for secretion. If this hypothesis is true, then changes in the uptake or metabolism of administered hormones could be important elements regulating the prostatic response to androgens. For example, the slower proliferative response induced by testosterone could be due to time required for induction of 5alpha-reductase to convert it to dihydrotestosterone, and the switch of the prostate from proliferation to secretion once the normal cellular complement has been restored might be a result of changes in the intracellular concentration of the various androgen metabolites. The model systems developed in Chapters III and IV can be used to examine changes in hormone uptake and metabolism following

castration and at various stages of regeneration.

Information accumulating about specific protein receptors for steroid hormones indicates that they are involved in transport of the hormone into the nucleus where it can interact with chromatin (Rennie and Bruchovsky, 1973). Thus these receptors may be a part of a mechanism whereby any changes in uptake and metabolism of hormone might be effected. The model system developed offers an opportunity to study whether changes in proliferative and functional status of the prostate are accompanied by changes in the receptors.

The interaction of steroids with chromatin is a subject that promises to yield much valuable knowledge about regulation of gene activity in general. Chromatin from unstimulated cells and from cells that are proliferating or differentiating can be compared, and the specific components of chromatin with which radioactive steroids interact can be determined.

ROLE OF ANDROGENS IN MAINTENANCE OF THE NORMAL PROSTATE

The process of prostatic atrophy appears to be characteristic of degenerative processes in general in that it involves loss of cells by autodigestion, but different from atrophic processes in tissues such as muscle which do not involve loss of cells but rather result from decreases

in cell size. Prostatic atrophy is an active process and not due merely to a cessation of cell production accompanied by continuation of normal turnover. Cell production does stop almost immediately (Chapter III), but the subsequent loss of cells is much more rapid than would be expected from normal turnover. Autolysis of cells is thought to be caused by the action of degradative enzymes, such as nucleases and proteases, normally confined to the lysosomes, which apparently are released when the level of androgens drops following castration. Thus the simplest interpretation of the role of androgens in preventing prostatic atrophy is that they repress the synthesis and/or activation of lysosomal degradative enzymes.

However the situation must be more complex than a simple repressive effect of androgens on lysosomes, since if this were the case all the cells should disappear following castration, but, as shown in Chapter III, this does not occur. One means by which survival of the prostate could be accomplished would be the existence of a specific subpopulation of cells in the normal prostate whose integrity does not depend on the presence of androgens. An attempt was made in Chapter III to test this possibility by determining whether cells in the normal prostate that can incorporate labeled thymidine can preferentially survive castration, but results were negative. Another possible characteristic that might distinguish such a putative

subpopulation would be the way it handles androgens, such as differences in receptors or metabolizing enzymes. This could be studied by following enrichment or depletion of these substances in the surviving cell population during the course of castration. Even if the postulated surviving population does exist, it does not escape entirely the effects of androgen deprivation, since the cells surviving castration have lost most of their cytoplasm and their functional capacity. Thus a limited amount of autolysis must have occurred in these cells. Hence a second possibility is that survival of a small number of cells, albeit functionally inactive cells, could be due to a requirement for continued production of lysosomal enzymes in order for autolysis to proceed. Since protein synthesis ceases after castration, the cells that have not been completely digested when degradative enzymes are depleted might be the ones to survive castration. A variation of this possibility would be the existence of a diffusible factor required for lysosome function secreted by the prostatic cells, acting on the tissue as a whole, and antagonized by androgens. When the number of cells had declined to a level below which subthreshold amounts of this factor were produced, autolysis would stop. The latter two possibilities predict that loss of cells following castration is random, while the first predicts that there is a subpopulation in the normal prostate predestined to survive castration.

REGULATION OF PROSTATIC PROLIFERATION AND DIFFERENTIATION

The initiation of DNA synthesis in the 7 day castrate prostate after hormone restoration is an event with a closely defined time course (Chapter IV). At 24 hours no DNA synthesis is detectable, while at 36 hours incorporation of thymidine is significantly elevated above control levels, and by 48 hours it is almost 100 times the control level. Thus the events occurring between 24 and 36 hours are probably the key to regulation of initiation of DNA synthesis. Cells can be compared at various times between 0 and 48 hours after initiation of treatment and appearance of stimulatory factors or disappearance of inhibitory factors could be measured with a suitable assay system. Detailed studies of RNA and protein synthesis and changes in chromatin during this period would undoubtedly provide valuable information about the processes involved in converting a quiescent cell population to a proliferating one.

The kinetics of cellular proliferation during the regenerative process, as studied in Chapter V, enable one to eliminate several possible mechanisms that might be involved in limiting the size of the prostate. Firstly, there does not appear to be a small stem cell population that cycles continuously, but rather all cells that survive castration probably proliferate at some time during regeneration. Secondly, proliferation does not seem to be limited by

depletion of some essential substance after a given number of divisions, since any given cell can apparently divide from one to four times. This conclusion is supported by the results with 4 day castrates reported in Chapter IV. In this case, if the cells underwent the same number of divisions as do those in 7 day castrates, then the result would be a hyperplastic prostate; however, this is not observed.

The cessation of proliferation does not occur because of loss of hormonal effects, since the prostatic weight continues to increase dramatically long after the number of nuclei has stopped increasing (Chapter IV). This shift in the response from proliferation to secretion must be due to some changes in the cells themselves. As discussed above, one type of change that could be envisaged would be an alteration in the manner in which cells take up and metabolize hormone. However, all results in Chapter IV point to the number of cells in the prostate as the key factor determining whether the response is proliferation (number of cells below normal) or secretion (number of cells normal). It is difficult to see how this type of regulation could be accomplished without some sort of mechanism dependent on total cell number. A negative feedback type of control, such as the chalone hypothesis of Bullough et al (1967), would be the simplest mechanism that could link the response to total cell number. If functional prostatic cells produced an inhibitor of proliferation such that the amount produced by

the normal number of cells were just sufficient to shut off proliferation, then proliferation would occur only when the number of cells had fallen below the normal level, and would be shut off in a regenerating population once the normal cellular complement had been restored, in accordance with the experimental observations. This key regulatory mechanism would be independent of hormone, although one of the means by which it might control proliferation could be by affecting the uptake and metabolism of hormone. In effect, then, there would be two superimposed mechanisms regulating prostatic size, one internal (feedback inhibitor) and one external (androgen). Androgens would be necessary but not sufficient for proliferation.

The response of the prostate to androgens is also an excellent system in which to study the relationship between proliferation and differentiation. Some indirect evidence indicating that secretion might not occur before proliferation is complete can be found in the studies in Chapter IV on the effects of testosterone on stimulating prostatic growth in 7 day castrates. Wet weight is not stimulated to increase at a rate greater than the rate of increase in numbers of nuclei. If the hypothesis of Robel et al (1971) that testosterone has more marked effects on secretion than on proliferation is correct, then this finding suggests that secretion cannot be stimulated until proliferation has been completed. Since the differentiated

functions of the prostate have been quite well characterized, it is possible to assay biochemically for functional capacity, rather than relying on wet weight as an indicator, as in the present study. Such studies could determine directly whether differentiation occurs simultaneously with proliferation, or whether proliferation must be completed before secretion commences. This distinction has important implications for the model of regulation of prostatic proliferation proposed above. If secretion were found to be stimulated prior to restoration of the normal cellular complement, then androgens could be considered to be the sole regulators of prostatic function, and it would be unnecessary to propose a superimposed internal control mechanism such as appears to be necessary for regulation of proliferation. If, on the other hand, secretion were not stimulated until proliferation had been completed, then it would appear that an internal control mechanism complementary to the one regulating proliferation might exist.

In summary, the dependence of the proliferative response of the prostate primarily on the size of its cellular complement and secondarily on the presence of androgen indicates that the potential of the prostate for proliferation is determined by some internal control mechanism based on the total number of cells present. Androgen is necessary for expression of this potential but

cannot override the internal control mechanism. Prostatic secretory function may depend exclusively on the presence of androgen or it may also be governed by some internal mechanism linked to the one regulating proliferation. It is possible that in prostatic carcinoma or benign prostatic hyperplasia the cause of the abnormal growth may be a defect in the internal control mechanism that fails to shut off the proliferative response to hormone.

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APPENDIX

Publications Arising from this Research:

Abstracts

LESSER, B. and BRUCHOVSKY, N., 1972. Regulation of Prostatic Growth by Androgens. Proceedings of the Canadian Federation of Biological Societies 15:413.

LESSER, B. and BRUCHOVSKY, N., 1972. Androgenic Regulation of Prostatic Growth and Function. Clinical Research 20:921.

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Articles

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BRUCHOVSKY, N., RENNIE, P. and LESSER, B., 1974. Mechanisms that Regulate the Concentration of Androgens in Nuclei of Prostatic Cells. Fourth International Congress on Hormonal Steroids, to be published.